Silencing, Heterochromatin, and DNA Double Strand Break Repair in *Saccharomyces cerevisiae*

by
Kevin David Mills

B.A. Molecular Cellular and Developmental Biology
University of Colorado, Boulder

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Signature of Author

Department of Biology

Certified by

Leonard Guarente
Professor, Department of Biology

Accepted by

Alan Grossman
Chairman, Biology Graduate Committee

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Abstract

The repair of damaged DNA is a crucial function carried out by all cells. Unrepaired DNA damage can lead to mutation, loss of genetic information, cellular transformation, or cell death. The DNA double strand break (DSB) is one type of damage that must be efficiently and accurately repaired. Two general mechanisms are employed by eukaryotic cells to repair DSBs: homologous recombination and nonhomologous end-joining (NHEJ). Much has been learned about both modes of DSB repair from studies in the budding yeast, *Saccharomyces cerevisiae*.

Recent studies in *S. cerevisiae* have implicated heterochromatin factors in NHEJ. In particular, the silent information regulatory genes *SIR2, SIR3,* and *SIR4*, required for telomere and mating-type silencing, are also required for efficient NHEJ. To better understand how telomere silencing and heterochromatin is established and regulated, a screen for high copy antagonists of telomere silencing was performed. One gene identifed in this screen, the anti-silencing factor gene *ASF1*, antagonizes telomere silencing and confers DNA damage resistance.

Telomeric heterochromatin factors directly respond to DSBs. Sir3p, required for NHEJ, redistributes from telomeres to DSBs following induction of breaks using the restriction endonuclease, *EcoR*I. This redistribution occurs specifically during the S phase of the cell cycle and requires components of the DNA damage checkpoint pathway. In addition, *ASF1* is required for NHEJ, interacts in a complex with Sir2p, and may participate in recruitment of heterochromatin factors to sites of DNA damage. A model for heterochromatin reorganization in response to DNA damage is discussed

Thesis supervisor: Dr. Leonard Guarente
Title: Professor, Department of Biology

I dedicate this work to Melissa, whose patience is infinite and whose love is invaluable;
and to my parents, Barbara and Ken Mills, who always said "Do whatever makes you happy" and supported me in so many ways through everything.

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Chapter 1: Introduction to silencing, heterochromatin, and DNA break repair

Position dependent repression of transcription is a common regulatory theme in eukaryotes. This phenomenon was first recognized in *Drosophila melanogaster* when chromosomal translocations that juxtaposed heterochromatic regions and euchromatic regions resulted in variegated expression of genes at the translocation junctions (reviewed in Weiler and Wakimoto, 1995). Modifiers of *Drosophila* position effect variegation include the chromatin factors HP1 and Polycomb group protiens (Stankunas et al., 1998; Sinclair et al, 1998; Clark and Elgin, 1992; Paro and Hogness, 1991; James et al., 1989). Gene silencing in vertebrate cells is often associated with genomic imprinting. The mouse H19 gene is expressed from the maternal chromosome only (Hark and Tilghman, 1998; Ishihara et al., 1998). On the paternal chromosome, a region near the H19 gene is highly methylated at CpG residues leading to transcriptionally repressive chromatin formation (Svensson et al., 1998; Tremblay et al., 1995). Gene silencing also affects the expression of transgenes in mammalian cells (reviewed in Henikoff, 1998). The insertion of tandem, multiple-copy tansgenes often leads to variable expression. Introduction of high copy number repeats results in extensive methylation and chromatin compaction within the transgenic sequence, leading to low level expression (Garrick et al., 1998).

YEAST SILENCING

Transcriptional silencing in *Saccharomyces cerevisiae* is associated with at least three regions of the genome: the cryptic mating type loci *HML* and *HMR* (Figure 1A), telomeres (Figure 1B), and the tandemly repeated

array of ribosomal RNA genes (rDNA) (Figure 1C). Silencing at *HM* loci and telomeres is best understood and requires many of the same factors. At *HM* loci, discrete silencer elements are required for the establishment and the maintenance of epigenetically stable silencing (reviewed in Lauenson and Rine, 1992). In contrast, genes placed near telomeres infrequently switch between silent and active states, and the new transcriptional state is epigeneteically maintained in daughter cells through several successive generations (Gottschling et al, 1990). Silencing in the rDNA is less well understood, but requires at least one factor necessary for *HM* and telomere silencing (Bryk et al., 1997; Smtih and Boeke, 1997; Fritze et al., 1997). Silent heterochromatin at the rDNA may be related to the structure of the nucleolus.

Silent *HM* loci

Haploid yeast cells normally exist as either mating type **a** or mating type α. Cell type is determined by the allele of the mating type genes located at the *MAT* locus. Wild type haploid cells can undergo mating type interconversion by replacing the genes at the MAT locus with the opposite mating type genes. During mating type switching the *HM* loci serve as donors for gene conversion (Rine and Heskowitz 1980; Klar, 1980; Strathern and Herskowitz, 1979; Klar and Fogel, 1979; Klar et al 1979). The arrangement of *MAT, HMR,* and *HML* on chromosome III is diagrammed in Figure 1A. Normally, *HMR* contains all of the information necessary to specify the **a** mating type and *HML* contains all the information necessary to specify the α mating type (Nasmyth and Tatchell, 1980; Hicks et al., 1979). However, only the genes found at the *MAT* locus are expressed because

HML and *HMR* are transcriptionally silenced (Abraham et al., 1984). Silencing of *HM* loci is dependent on several DNA sequence elements that constitute silencers flanking each locus (Feldman et al., 1984). The best characterized of these is the HMR-E silencer required for silencing of *HMR* (Brand et al., 1985). This silencer consists of three distinct DNA elements: A, E, and B. (McNally and Rine, 1991; Brand et al, 1985). The A element contains an Autonomously Replicating Sequence (ARS) consensus bound by the Origin Recognition Complex (ORC), the E element contains a binding site for the Repressor/Activator Protein 1 (Rap1p), and the B element contains a binding site for the ARS binding factor 1 (Abf1p) (Rivier and Rine, 1992; Buchman et al., 1988; Kimmerly et al, 1988; Buckman Lue and Kornberg, 1988). Any one of these silencer elements is dispensable for silencing, but elimination of any two completely abrogates silencer function.

SIR complex

Silencing requires the products of *SIR1*, *SIR2*, *SIR3*, and *SIR4* (Ivy et al.,1986). *SIR1* is required for the establishment, but not the maintenance, of *HM* silencing, and has no known function at telomeres (Aparicio et al, 1991; Pillus and Rine, 1989; Ivy et al., 1986). Wild type haploid cells retain *HM* silencing, and therefore mating proficiency, with extremely high efficiency. Mutation of *SIR1* results in derepression of *HM* loci and sterility in approximately 80 percent of the cells in a population (Rine et al., 1979). Infrequently, *sir1* cells switch from sterile to fertile, indicating that silencing has been reestablished at *HML* and *HMR* (Pillus and Rine, Cell 59:637). Once the state of silencing in a *sir1* strain has switched, it is stably maintained in that cell lineage for several generations before switching

again. Maintenance of silencing in *SIR1* or *sir1* cells requires *SIR2*, *SIR3*, and *SIR4*. Mutation of *SIR2*, *SIR3* or *SIR4* results in a complete loss of silencing at *HM* loci as well as telomeres(Aparicio et al., 1989; Nasmyth, 1982; Shore et al, 1984; Rine and Herskowitz, 1987). Immunoprecipitation and two-hybrid analyses indicate that Sir2p, Sir3p and Sir4p interact in a complex (Moretti et al., 1994; Moazed et al., 1997; Gotta et al., 1998). Sir3p and Sir4p also interact with Rap1p (Moretti et al., 1994) and Sir3p interacts with histones H3 and H4 (Venditti et al., 1999; Hecht et al.,1996; Hecht et al, 1995).

Immunolocalization/in situ hybridization shows Sir3p and Sir4p to be concentrated in discrete foci coincident with clustered telomeres (Gotta et al. 1996; Maillet et al., 1996; Cockell et al., 1995; Palladino et al., 1993) while Sir2p concentrates in the nucleolus as well as at telomeres (Shou et al., 1999; Straight et al., 1999; Gotta et al., 1997). These observations support a model of silencing in which complexes of Sir proteins polymerize through interactions with Rap1p and histones to form transcriptionally inert heterochromatin. This heterochromatin is highly dynamic and is regulated by environmental conditions. Stimulation of cells with mating pheromone, heat shock, or starvation causes hyperphosphorylation of Sir3p by the mitogen activated protein (MAP) kinase pathway (Stone and Pillus, 1996), resulting in increased telomere silencing.

Cellular aging also causes reorganization of *SIR*-dependent heterochromatin (Kennedy et al., 1997). Yeast cells exhibit a finite replicative lifespan and undergo characteristic phenotypic changes as they age(Mortimer and Johnston, 1959; Muller, 1971; Barton, 1950). One such change is the occurrence of sterility in haploid cells beginning after

approximately 50 percent of the lifespan has been completed (Muller, 1985; Smeal, et al, 1995). This age-related sterility is due to a loss of silencing at *HM* loci and is associated with redistribution of Sir3p from telomeres and *HM* loci to the nucleolus (Kennedy et al., 1997).

RAP1

HM and telomere silencing also requires the sequence-specific DNA binding protein encoded by *RAP1*. Rap1p is an essential protein with structural and functional similarity to Abf1p (Diffley and Stillman, 1989). In addition to its role in silencing (Shore and Nasmyth, 1987) Rap1p activates transcription of glycolytic and ribosomal protein genes (Moehle and Hinnebusch, 1991; Freeman et al., 1995; Scott and Baker, 1993; McNeil et al., 1990; Chambers et al., 1990; Chambers et al., 1989). Although *RAP1* is essential, missense mutations within a small region of the C-terminus are viable (Moretti et al., 1994; Kyrion et al., 1993). C-terminal mutations in Rap1p result in diminished interaction with Sir3p, crippled *HM* and telomere silencing, telomere elongation and elevated levels of chromosome non-disjunction (Moretti et al., 1994; Liu, et al., 1994; Kyrion et al., 1993; Hardy et al., 1992; Sussel and Shore, 1991). This indicates that Rap1p plays a central role in silencing and telomere maintenance and suggests that these functions occur through interactions with the Sir complex.

ORC

A variety of other protein factors modify, stabilize, or coordinate silencing. These include the origin recognition complex (ORC) histones H3 and H4, and the something about silencing (*SAS*) genes. ORC is an

essential, six subunit complex that binds to ARS elements and is necessary for initiation of replication (Bell and Stillman, 1992; Diffley and Cocker, 1992). ARS elements are distributed throughout the genome and serve as origins of DNA replication initiation (Huang and Kowalski, 1996; Deshpande and Newlon, 1992; Brewer and Fangman, 1987; Tanaka et al., 1996). ORC binding to ARS elements recruits minichromosome maintenance (MCM) proteins into a pre-replication complex (pre-RC) prior to intitation of DNA synthesis (Aparicio et al, 1997; Tanaka et al., 1997). After an origin has fired, MCM proteins dissociate from origin DNA and associate with non-origin DNA at replication forks (Aparicio, et al., 1997). Once an origin has fired, it does not normally re-initate replication until after M-phase and is thought to be controlled by recruitment of MCM proteins into the pre-RC by ORC (Aparicio et al., 1997; Drury et al., 1997; Donovan et al., 1997). ORC is also required for efficient silencing at *HM* loci (Micklem et al., 1993; Foss et al., 1993; Bell, et al., 1993; Fox et al., 1995; Loo, et al., 1995). Passage through the S-phase of the cell cyce is necessary to reestablish *HM* silencing that has been disrupted in a *sir3* temperature sensitive strain (Triolo and Sternglanz, 1996; Laman et al., 1995). This suggets that silencing is linked to DNA synthesis. This idea is further supported by the presence of ARS elements within *HM* silencers (McNally and Rine, 1991; Brand et al., 1987). Mutations in the HMR-E ARS that abolish origin activity also result in crippled silencing (Sussel et al., 1993; Mahoney et al., 1991). Two observations support the idea that ORC may recruit Sir1p to *HM* silencers. First, Orc1p can bind Sir1p and second, tethering Sir1p directly to silencers eliminates the ORC requirement

(Gardner et al., 1999; Fox et al., 1997; Tiolo and Sternglanz, 1996; Chien et al., 1993).

Histones

Silent heterochromatin is thought to repress transcription by restricting access of transcriptional machinery to the chromatin template. Compaction of silent heterochromatin may occur through interactions with nucleosomes. Nucleosomes are sequence independent DNA binding octamers composed of two molecules each of histone H2A, H2B, H3, and H4 (reviewed in Fletcher and Hansen, 1996). Numerous findings implicate histones in transcriptional silencing. Mutations that change basic residues in the N-terminus of histone H4 result in derepression of *HM* loci, and can be suppressed by mutations in *SIR3* (Johnson et al., 1990). Sir3p and Sir4p have been shown to physically interact with the N-termini of histones H3 and H4 (Johnson et al., 1990; Hecht et al., 1995; Venditti et al., 1999). Mutations in *SIR3* and *SIR4* that abolish interaction of the proteins with histones also confer a silencing defect, confirming the functional importance of Sir-histone interactions (Hecht et al.,1995).

Telomere silencing

Telomeres also exert transcriptional repression on genes placed near chromosome ends (Gottschling et al., 1990). Telomeric silencing, unlike *HM* silencing, is unstable and is able to spread inward along the chromosome up to several kilobase pairs from the chromosome end (Pryde and Louis, 1999; Aparicio et al., 1994; Renauld et al., 1993; Gottschling et al., 1990). Genes near telomeres frequently switch between silent and active

states (Monson, et al., 1997; Gottschling et al., 1990). Similar to silencing at *HM* loci in a *sir1* mutant, the state of silencing at a telomere that has switched persists for several generations within a cell lineage until the next switch (Gottschling et al., 1990). Telomeric silencing requires *RAP1*, *SIR2*, *SIR3*, and *SIR4* (Aparicio et al., 1991; Kyrion, et al., 1993; Liu et al., 1994). Telomere silencing is highly sensitive to the dosage of Sir proteins. Overexpression of Sir2p can substantially increase telomere silencing (see Chapter 2), while overexpression or Sir4p inhibits telomere silencing (Gotta et al., 1998; Smith et al., 1998) . Overexpression of Sir3p can either inhibit telomere silencing or enhance chromatin spreading, depending on strain background, dosage, and assay conditions (Strahl-Bolsinger et al., 1997; Gotta et al., 1998; Renauld et al., 1993). Although *SIR1* normally plays no role in telomere silencing, tethering of Sir1p near a telomere is sufficient to stabilize telomere silencing (Chien et al., 1993). Telomere silencing also requires *HDF1* and *HDF2*, encoding the 70 and 80 kD subunits of the Ku DNA end-binding heterodimer (Boulton and Jackson, 1998; Laroche et al., 1998). Other telomere associated proteins that affect silencing include the Rap1p interacting factor Rif1p (Wotton and Shore, 1997; Marcand et al., 1996; Sussel et al., 1995; Hardy et al., 1992), and the single stranded DNA binding protein Cdc13p (Nugent et al., 1998; Lin and Zakian, 1996; Nugent et al., 1996; Garvik et al., 1995). The involvement of DNA repair proteins in telomeric silencing and maintenance suggests that repressive heterochromatin at telomeres provides multiple functions. In addition to transcriptional silencing, telomeric heterochromatin may serve to repress telomere recombination and could act as a reservoir for DNA repair factors (see below).

Telomere structure

One particular challenge for eukaryotic cells is maintenance and replication of chromosome ends. Without a specialized mechanism of chromosome end maintenance, the DNA molecule would shorten with each successive round of replication. This could eventually lead to deletion of essential genes or loss of the chromosome. In addition, the ends of linear chromosomes are potential substrates for DNA repair machinery dedicated to the repair of DSBs. This could lead to end-to-end chromosome fusions and genome instability. However the specialized structure of telomeres ensures proper replication and maintenance of linear chromosome ends.

In *Saccharomyces*, telomeres are composed of a repeating TG_{1-3} sequence approximately 300 bp in length (Walmsley et al., 1984; Shampay et al., 1984). Telomere tract length in yeast is distributed over a very narrow range (Marcand et al., 1997; Lustig et al., 1990) and is maintained by the ribonucleoprotein reverse transcriptase, telomerase, encoded by *EST1* and*TLC1* (Virta-Pearlman, et al., 1996; Steiner et al, 1996; Cohn and Blackburn, 1995; Lin and Zakian, 1995; Singer and Gottschling, 1994). Centromere-proximal to the G rich terminal sequences are two classes of subtelomeric repetitive sequences. Almost all chromosomes contain consensus repeats termed X-elements (Louis, 1995; Louis et al., 1994). Some, but not all, chromosomes also contain a highly polymorhpic repeat termed the Y'-element (Louis, 1995; Louis et al., 1994; Zakian et al., 1986; Jager and Philippsen, 1989). Like *HM* silencers, X-elements contain ARS consensus sequences and most X-elements also contain a binding site for Abf1p (Louis, 1995; Louis et al., 1994). The function of the polymorphic Y' is unknown, but may be involved in telomerase independent chromosome

end maintenance (Louis and Haber, 1992). The repetitive nature of telomeric DNA presents cells with the additional challenge of preventing inappropriate homologous recombination between telomeres that could result in end-to-end chromosome fusions. This may be achieved partially by the formation of a heterochromatin structure at telomeres that prevents access by DNA repair machinery. Paradoxically, this repressive telomeric chromatin structure in yeast is dependent on elements of the NHEJ machinery, which are discussed in detail below.

In strains lacking functional telomerase, most cells exhibit rapid telomere shortening followed by senescence (Lendvay et al., 1996; Lundblad and Szostak, 1989). However, a small fraction of cells survive telomere shortening and maintain chromosome ends by homologous recombination (Le et al., 1999; Lundblad and Blackburn, 1993). Telomerase-independent telomere maintenance requires the *RAD52*-dependent recombination pathway (Le et al., 1999). In telomerase deficient cells that survive senescence, Y'-elements become amplified and a helicase (Help1) encoded in the Y'-element becomes expressed (Yamada et al., 1998). The function of Help1 is unknown, but may facilitate homologous recombination between chromosome ends.

Human telomeres are composed of approximately 5kb of repeating TTAGGG sequences (Luderus et al., 1996; deLange et al., 1990; Morin, 1989; Cross, et al., 1989). Recent studies have indicated that human telomeres assume a characteristic conformation, termed a T-loop (Griffith et al., 1999). T-loops are thought to form when single stranded DNA at the end of the chromosome invades telomeric DNA at a more centromere-proximal position. This structure is thought to confer stability on the ends,

in part by occluding DNA repair machinery from binding the end. In normal somatic cells telomerase is inactive and telomeric tracts shorten with successive rounds of DNA replication. Shortening of telomeres beyond a critical minimum length in human cells induces cellular senescence. Re-activation of the catalytic subunit of telomerase, hTERT, is sufficient to immortalize cells in culture (Bodnar et al., 1998; Counter et al., 1998; Nakayama et al., 1998; Weinrich et al., 1997; Meyerson, et al., 1997).

Telomeric DNA tracts in mice can be as long as 25 kb and are actively maintained by telomerase activity. Mice deficient for the RNA component of telomerase (mTR) exhibit progressive telomere shortening at a rate of approximately 5kb per generation (Blasco, et al., 1997). Starting at generation four, cells derived from mTR-/- mice show significant telomere shortening, aneuploidy and chromosome rearrangements. By the sixth generation telomerase deficient mice display significant infertility and severe developmental defects (Rudolph et al., 1999; Niida et al., 1998; Blasco et al., 1997).

DNA DAMAGE REPAIR

The maintenance of chromosome stability and integrity is crucial to all cells. Therefore cells utilize elaborate and efficient mechanisms to repair damaged DNA. One particularly detrimental type of DNA lesion is the double strand break (DSB). Failure to properly repair a DSB can result in loss of genetic information, mutagenesis, or lethality. Most eukaryotic cells employ two general strategies to repair DSBs: homologous recombination,

and non-homologous end-joining (NHEJ). The combination of these strategies ensures that DSBs are rapidly and accurately repaired.

A great deal has been learned about eukaryotic DNA repair by the study of the budding yeast *Saccharomyces cerevisiae*. Recent studies have directly implicated heterochromatin factors in DSB repair. Interestingly, many DSB repair factors also participate in telomere maintenance and telomeric heterochromatin formation. These reciprocal findings lead to a picture of regulated telomeric heterochromatin poised to respond to DNA damage by mobilizing repair machinery.

The work detailed in this thesis is primarily concerned with the involvement of heterochromatin factors in DSB repair. However, I provide a general overview of both homologous recombination and nonhomologous end-joining below.

Homologous recombination

DNA repair by homologous recombination occurs when a lesion is corrected by strand transfer, replication, and resolution from an undamaged, homologous DNA molecule. Repair by homologous recombination ensures the conservative, accurate repair of many types of DNA lesions, including DSBs.

The various enzymatic steps required to carry out homologous recombination are most well defined in bacteria and are shown schematically in Figure 2 (reviewed in Eggleston and West, 1996; Camerini-Otero and Hsieh, 1995). These steps include DNA strand invasion to form a heteroduplex, extension of the heteroduplex, with associated branch migration of the DNA cross-over, DNA replication, cleavage of the Holliday

junction, and ligation of the resulting nicked DNA. This process involves at least 25 different enzymatic functions. Recombination is typically initiated by the RecBCD enzyme, which unwinds and degrades double stranded DNA (Farah and Smith, 1997; Taylor and Smith, 1995; Dixon and Kowalczykowski, 1995; Roman and Kowalczykowski, 1989). The RecA protein then stimulates recombination by forming a nucleoprotein filament along single stranded DNA, promoting homologous DNA pairing and strand exchange (Gupta et al., 1998; Aihara et al., 1997; Bazemore et al., 1997; Hsieh et al., 1992; Chow et al., 1992; Conley and West, 1989; Honigberg, et al., 1985). The invading single strand is used as a primer to initiate DNA replication, filling in deleted sequence (Asai et al., 1994). Branch migration is carried out by RuvAB (reviewed in West, 1997). RuvB is a DNA helicase loaded onto the Holliday junction by RuvA (Seigneur et al., 1998; Nishino et al., 1998; Yu et al., 1997; Whirtby et al., 1996; Mitchell and West, 1996). To complete DNA repair, the Holliday junction must be resolved. This is accomplished by the RuvC endonuclease. RuvC interacts with RuvB present at the junction, recognizes the crossover, and introduces single strand nicks in the duplex DNA near the junction (Zerbib et al., 1998; Benson and West, 1994; Sharples and Lloyd, 1991; Connoly et al., 1991). The crossover is then separated and the single strand nicks are ligated to yield two intact DNA duplexes.

Homologous recombination in yeast requires DNA repair genes in the *RAD52* epistasis group. This group includes *RAD51*, *RAD52*, *RAD54*, *RAD57*, and *RAD59*. *RAD52* was cloned by complementation of the methyl methane sulfonate and ionizing radiation sensitivity of a *rad52-1* mutant strain (Adzuma et al., 1984). *RAD52* encodes a 56kD protein that is

structurally and functionally conserved from yeast to humans (Bezzubova et al., 1993; Muris et al., 1994). Mutation of *RAD52* in yeast results in hypersensitivity to ionizing radiation (Resnick and Martin, 1976; Ho, 1975) and radiomimetic drugs (Prakash and Prakash, 1977), defects in mating type interconversion (Malone and Esposito, 1980), and decreased recombination rate within repeated DNA (Rattray and Symington, 1994; Fan and Klein, 1994; Thomas and Rothstein, 1989). During DSB repair by homologous recombination the Rad52 protein binds to broken ends, protects them from nucleolytic degradation (Van Dyck, et al., 1999), and stimulates the association of Rad51p with single stranded DNA (New et al., 1998; Sugiyama et al., 1998; Donovan et al., 1994; Milne and Weaver, 1993). *RAD51*, a homologue of *E. coli* recA, encodes a sequence independent DNA binding protein that forms a nucleoprotein filament on single stranded DNA to initiate homology pairing (Ogawa et al., 1993; Shinohara et al., 1992). Rad51p cooperates with the double stranded DNA-dependent ATPase, Rad54p, to promote DNA strand invasion, , and form the heteroduplex DNA necessary for recombination (Petukhova et al., 1998; Clever et al., 1997).

Rad55p and Rad57p, also RecA homologues, form a heterodimer that interacts with both Rad51p and the single stranded DNA binding protein replication protein A (RPA) to accelerate the DNA strand exchange reaction (Gasior et al., 1998; Sung, 1997), perhaps by alleviating competition for ssDNA between RPA and Rad51p (Sung et al., 1997). Holliday structures are resolved, as in *E. coli*, when a specific endonuclease recognizes and cleaves the junction (Parsons and West, 1988; Evans and Kolodner, 1987; Symington and Kolodner 1985).

Yeast cells possess two biochemically distinct DNA ligase activities (Ramos et al, 1997), DNA ligase I (Johnston and Nasmyth, 1978) and DNA ligase IV (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997). The yeast DNA ligase I, encoded by *CDC9* (Johnston and Nasmyth, 1978), is the major DNA joining activity is yeast and is required for normal DNA replication as well as homologous recombination (Tomkinson et al., 1992; Johnston 1983; Fabre and Roman, 1979; Johnston and Nasmyth, 1978). DNA ligase IV, required for nonhomologous end-joining, is discussed below. Cdc9p can ligate single strand DNA nicks present on DNA:DNA duplexes and is implicated in mutagenic DNA repair when mismatches occur at nick sites (Tomskinson et al., 1992). Cdc9p is necessary to complete homologous recombination by ligating nicks resulting from the resolution of Holliday junctions (Tomkinson et al., 1992).

Single strand annealing

An alternative mode of DNA repair to the conservative homologous recombination, is single strand annealing (SSA) (Haber, 1995). SSA can occur when there is homology between DNA sequences flanking a DSB. Unwinding of double stranded DNA on either side of the break is followed by annealing of single stranded DNA on one side of the break to its complementary sequence on the other side of the break (Fishman-Lobell et al., 1992; Fishman-Lobell and Haber, 1992). In *Saccharomyces*, annealing is promoted by the recombination factor Rad52p (Prado and Aguilera, 1995; Ivanov et al., 1996). When this results in double stranded DNA containing single stranded 3' "tails", the non-base paired tails must be removed before recombination can be completed. Long tails are cleaved by the nucleotide

excision repair endonuclease Rad1p/Rad10p (Davies et al., 1995; Ivanov and Haber, 1995; Prado and Aguilera, 1995). Cleavage of short nonhomologous tails also requires the base mismatch repair proteins, Msh2p/Msh3p (Sugawara et al., 1997). Removal of the single strand tails can result in the deletion of that DNA and mutagenesis.

Post replication repair

In addition to the error free, conservative homologous recombination pathway, yeast cells possess post-DNA replication repair mechanisms that can be either error-free or error-prone. This pathway is involved in mutagenic DNA repair by translesion DNA synthesis, spontaneous mutagenesis, and the maintenance of DNA sequence repeat stability.

The *RAD6* epistasis group

RAD6, which defines the post-replication repair epistasis group, is required for post-replication repair of UV damage, DNA damage induced mutagenesis (Cassier-Chauvat and Fabre, 1991; Prakash, 1989), ubiquitin-dependent protein degradation (Bailly et al., 1994) , and telomeric silencing (Huang et al., 1997). Rad6p physically interacts in separate complexes with the ubiquitination factor Ubr1p and with the DNA repair protein Rad18p (Bailly et al., 1997; Bailly et al, 1994). DNA replication past unrepaired, UV-induced pyrimidine dimers is base-mismatch tolerant and requires *RAD6* and *RAD18* (Roche et al, 1995). Mutations in *RAD6* or *RAD18* result in UV hypersensitivity and a reduced level of UV induced mutagenesis (Cassier-Chauvat and Fabre, 1991), but an increased rate of spontaneous mutagenesis (Roche et al., 1995). These findings suggest that the

Rad6p/Rad18p complex participates in both error-prone and error-free branches of the post-replication DNA repair pathway.

The *RAD5* recombination regulator

RAD5 encodes a DNA helicase with structural homology to the transcriptional regulator Swi2p (Richmond and Peterson, 1996; Johnson et al., 1994). *RAD5* is in the *RAD6* epistasis group and strains mutant for *RAD5* are sensitive to UV irradiation and DNA alkylating agents (Liefshitz et al., 1998; Glassner and Mortimer, 1994; Johnson et al., 1992). *RAD5* functions in an error-prone post-replication repair pathway parallel to *RAD18* and dependent on *RAD6*. Strains mutant for *RAD5*, like strains mutant for *RAD18*, show a decreased rate of UV-induced mutagenesis (Siede and Eckhardt, 1986; Siede et al., 1983). Also like *RAD18*, *RAD5*-dependent mutagenesis is dependent on trans-lesion DNA synthesis (Liefshitz et al., 1998). *RAD5* is also a regulator of spontaneous homologous recombination. *RAD5* mutant strains exhibit an increase in recombination rate in tandemly repeated DNA sequences, but a decrease in simple repeat instability (Liefshitz et al., 1998; Johnson et al., 1992). *RAD5* is also reported to be involved in the avoidance of DNA gap repair by NHEJ possibly by increasing the efficiency of homologous recombination near double stranded DNA gaps (Ahne et al., 1997). Rad5p is thought, therefore to be an accessory factor for homologous recombination that is involved in modulating efficiency of repair.

Nonhomologous end-joining

The other general mechanism of DSB repair is non-homologous end joining. Unlike homologous recombination, NHEJ does not depend upon homology to carry out repair. In contrast, broken ends are brought into proximity and ligated together to seal the break and produce a contiguous DNA duplex.

In yeast, NHEJ requires *HDF1* (yKu70), *HDF2* (yKu80) (Tsukamoto et al., 1996; Milne et al., 1996; Boulton and Jackson, 1996), *DNL4* (DNA ligase IV) (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997), *RAD50, XRS2, MRE11* (Tsukamoto et al., 1997; Milne et al., 1996; Moore and Haber, 1996) , *SIR2, SIR3*, and *SIR4* (Tsukamoto et al., 1997). *HDF1* and *HDF2* encode the 70 and 80 kD subunits of the yeast Ku end-binding heterodimer (Teo and Jackson, 1997; Feldmann et al., 1996; Tsukamoto et al., 1996; Mages et al., 1996; Porter et al., 1996; Siede et al., 1996). Ku is a sequence independent DNA binding heterodimer that recognizes ends of linear DNA molecules with high affinity (Blier et al., 1993). Rad50p, Xrs2p, and Mre11p participate in a multi-functional complex that has exonuclease activity (Chamankhah and Xiao, 1999; Usui et al., 1998; Ohta et al., 1998). The precise role of Rad50p/Xrs2p/Mre11p in NHEJ is not clear, but may produce single stranded DNA near DSBs that is necessary to initiate end-joining (Paull and Gellert, 1999). Alternatively, Rad50p/Xrs2p/Mre11p may function in the establishment or modification of a favorable chromatin structure at break sites. The requirement of *SIR2*, *SIR3*, and *SIR4* suggests that a heterochromatin-like structure may be necessary for efficient NHEJ (Tsukamoto et al., 1997). Finally, DNA ligase

IV, encoded by *DNL4* is required to religate the broken DNA molecule once properly processed ends are brought into juxtaposition (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997). In mammalian cells, NHEJ also requires the activity of DNA dependent protein kinase (DNA-PK) (Gu et al., 1998; Jhappan et al., 1997; Daza et al., 1996). The catalytic subunit of DNA-PK (DNA-PK_{cs}) is a member of the PI-3 kinase like family of proteins that includes human Atm and Atr (Hartley et al., 1995). Yeast members of this family include Mec1p and Tel1p (Morrow et al., 1995), but no functional homologue of DNA-PK_{cs} has been identified in yeast.

In *Saccharomyces*, homologous recombination is highly effective at repairing most types of DNA damage. However, defects in NHEJ machinery result in hypersensitivity to chemical agents that generate DSBs. NHEJ deficient cells are also highly sensitive to endonuclease mediated cleavage of the genome (Mills et al., 1999; Martin et al., 1999; Lewis et al., 1998).

In addition, *HDF1*, *HDF2* (Laroche et al., 1998; Nugent et al., 1998; Boulton and Jackson, 1998), *SIR2*, *SIR3*, and *SIR4* are required for normal telomere maintenance and silencing. *RAD50*, *XRS2*, and *MRE11* are also required for proper telomere structure maintenance, but not for telomere silencing (Le et al., 1999; Chamankhah and Xiao, 1999; Moreau et al., 1999; Boulton and Jackson, 1998). These findings indicate an integral relationship between DNA end joining and heterochromatin formation. How end joining factors catalyze DNA break repair at sites of damage, but protect chromosome ends from recombination or degradation remains a mystery.

In mammalian cells, NHEJ is an efficient mode of DSB repair, and NHEJ factors also participate in the specialized V(D)J recombination

pathway. Cells deficient for Ku70, Ku80, or DNA-PK_{cs} are severely compromised for DSB repair and are hypersensitive to ionizing radiation (Gao et al, 1998; Gu et al, 1997; Ouyang et al., 1997). Ku or DNA-PK_{cs} deficient mice also display severe combined immune deficiency (SCID) due to the failure to properly carry out T- or B- lymphocyte maturation (Gao et al., 1998; Gu et al., 1997). Mutations in *NBS1*, a member of the hMRE11/RAD50 DSB repair complex (Carney et al., 1998), or mutations in the DSB repair protein nibrin, cause Nijmegen break syndrome (NBS) Varon et al., 1998). NBS is characterized by a high incidence of cancer, hypersensitivity to ionizing radiation, and defects in cell cycle checkpoint control (Sullivan et al., 1997; Huo et al., 1994; Taalman et al., 1989; Taalman et al., 1983). Mice deficient in DNA ligase IV exhibit late embryonic lethality and fail to carry out V(D)J rearrangements (Frank et al., 1998). Fibroblasts derived from ligase IV deficient embryos show defects in growth and are hypersensitive to ionizing radiation (Frank et al., 1998). Mice deficient in the ligase IV interacting factor XRCC4 also show an absence of V(D)J recombination, and lethality at late embryonic stages accompanied by defects in neurogenesis (Gao et al., 1998). These findings suggest that normal developmental processes, as well as chromosome surveillance and stability activities, are dependent on NHEJ machinery and functional DSB repair.

Cell cycle checkpoint control

In addition to DNA repair mechanisms, cells must ensure proper coordination of DNA repair within the cell cycle. DNA lesions that are not repaired prior cell cycle progression can lead to mutagenesis. If mitosis

commences before repair of a DSB can take place, the cell risks loss of genetic information, with potentially lethal consequences. In higher eukaryotes this can lead to cellular transformation and cancer formation. Therefore cells must properly orchestrate DNA repair and cell cycle progression via checkpoint control. The major elements of the *Saccharomyces* DNA damage checkpoint pathway are diagrammed in Figure 4.

Cell cycle checkpoints monitor the status of events necessary for cell division, such as DNA replication, DNA repair, and spindle formation, and arrest cell cycle progression until the completion of these processes. Checkpoint machinery monitors DNA replication and repair during the S- and G2-phases of the cell cycle and prevents entry into mitosis until both have been completed. The yeast DNA damage checkpoint requires at least eight genes: *MEC1*, *RAD9*, *RAD53*, *RAD17*, *RAD24*, *MEC3*, *DUN1*, and *DDC1* (reviewed in Weinert, 1998).

RAD24 encodes a protein with structural similarity to the Replication Factor C (RFC). RFC is a pentameric DNA polymerase cofactor with DNA dependent ATPase activity that is stimulated by interaction with proliferating cell nuclear antigen (PCNA) and deposits PCNA on DNA (Cullman et al., 1995; Fien and Stillman, 1992). *RAD24* and RFC both participate in the S-phase DNA damage checkpoint (Paulovich et al., 1997; Sugimoto et al., 1997). Deletion of *RAD24* or point mutations in the gene encoding the small subunit of RFC (*RFC5*) result in reduced Rad53p phosphorylation in response to DNA damage and hypersensitivity to DNA damaging agents (Shimomura et al., 1998). Although null mutations in RFC subunits are lethal, point mutations in *RFC5* result in slow growth and a

defect in the DNA replication checkpoint (Sugimoto et al., 1997), as well as the DNA damage checkpoint. Rad17p, Mec3p, and Ddc1p appear to act downstream of Rad24p and transduce the signal to Mec1p (Kondo et al., 1999).

MEC1 is a member of the PI-3 kinase like family of genes that includes yeast *TEL1*, and mammalian *ATM*, *ATR*, and DNA-PK_{cs} (reviewed in Hoekstra, 1997). These genes all encode DNA damage responsive protein kinases. Mec1p, Tel1p, Atm, and Atr all function in DNA damage checkpoints, and DNA-PK_{cs} is necessary for NHEJ in mammalian cells. Mec1p and Tel1p respond to a variety of DNA lesions by phosphorylating Rad9p, to signal cell cycle arrest and transcriptional activation of DNA repair genes (Emili, 1998; Vialard et al., 1998; Sanchez et al., 1996; Paulovich and Hartwell, 1995; Weinert et al., 1994). *MEC1* is also required for cell cycle arrest when DNA replication is inhibited (Weinert et al., 1994). This checkpoint pathway is distinct from the DNA damage checkpoint pathway as it does not require *RAD9*. Deletion of *MEC1* is lethal, but this lethality can be suppressed by deletion of *SML1* (Zhao et al., 1998). *SML1* (Suppressor of *mec1* Lethality) encodes a negative regulator of the large subunit of ribonucleotide reductase, Rnr1p. Deletion of *SML1* results in elevated levels of cellular dNTP. This suggests that *MEC1* functions in normal cell growth by relieving *SML1*-dependent repression of RNR to elevate dNTP levels in preparation for DNA synthesis. Strains mutant for *MEC1* and *SML1* are viable but hypersensitive to replication blockage by hydroxyurea (HU), an inhibitor of RNR, and to a wide spectrum of DNA damaging agents, indicating that *mec1* mutants lack both the DNA replication and DNA damage checkpoints.

RAD9 is essential for the DNA damage checkpoint but is dispensable for the DNA replication checkpoint and for normal growth (Weinert and Hartwell, 1988). Rad9p contains a tandem repeat of a BRCA1 homology domain, called the BRCT domain. This sequence motif mediates protein-protein interactions and is found in a diversity of proteins, many of which are DNA damage responsive (Soulier and Lowndes, 1999; Yarden and Brody, 1999; Chai et al., 1999; Zhang et al., 1998). These include human BRCA1, XRCC1, XRCC4, DNA ligase III, DNA ligase IV, yeast DNA ligase IV, Rad9p, Lif4p, Rev1p, and Rap1p (Bork et al., 1997; Koonin et al., 1996). Deletion of *RAD9* results in no overt growth defect, but leads to hypersensitivity to ionizing radiation, UV, radiomimetic drugs, and deficient activation of DNA repair genes following treatment with these agents (Weinert and Hartwell; 1990; Elledge and Davis, 1990; Shiestl et al., 1989). Mutations in the Rad9p BRCT motifs can produce a null phenotype, indicating that these domains are necessary for Rad9p function (Soulier and Lowndes, 1999). Rad9p is hyperphosphorylated by Mec1p, Tel1p, and Rad53p in response to DNA damage (Emili, 1998; Vialard et al., 1998). Hyperphosphorylation of Rad9p stimulates self-association through the BRCT domains, as well as interaction with Rad53p (Soulier and Lowndes, 1999). This suggests that Rad9p functions as an oligomer following DNA damage and is involved in a complex phosphorylation circuit controlling cell cycle arrest and DNA repair.

RAD53 encodes an essential protein kinase containing a forkhead-homology associated (FHA) domain. The FHA domain of Rad53p is required for activation by association with phosphorylated Rad9p (Sun et al., 1998). Activated Rad53p causes cell cycle arrest in G2 and induces

expression of DNA repair genes (Navas et al., 1996; Sun et al., 1996; Allen et al., 1994). Rad53p delays S-phase following DNA damage by inhibiting transcription of the G1 cyclin genes, *CLN1* and *CLN2* (Sidorova and Breeden, 1997). Rad53p activates transcription of DNA damage inducible genes by inactivating the DNA binding regulator Crt1p (Huang et al., 1998). Crt1p recruits the Ssn6p/Tup1p repressor complex to damage inducible genes. Rad53 phosphorylation of Crt1p inhibits its binding to DNA, thus relieving Ssn6p/Tup1p-dependent repression.

DUN1 encodes a protein kinase that autophosphorylates and stimulates expression of *RNR1*, *RNR2*, and *RNR3* in response to DNA damage and replication blocks (Allen et al., 1994; Zhou et al., 1993). This results in elevated levels of deoxyribonucleotide necessary for DNA replication dependent repair and for recovery from replication blockage. Although replication blockage by hydroxyurea activates a Mec1p/Rad53p dependent DNA checkpoint, *RAD9*, *RAD24*, *RAD17*, *MEC3*, and *DDC1* are not required for this response, indicating that these factors are dedicated to DNA damage sensing.

The mammalian S-phase DNA damage checkpoint requires the gene mutated in the human syndrome ataxia telangiectasia (A-T). A-T is characterized by a predisposition to cancer, radiosensitivity, neurological and immunological defects, progressive ataxia, and infertility (reviewed in Canman and Lim, 1998). *ATM*, and its homologue *ATR*, encode PI-3 kinase-like proteins with homology to yeast Mec1p and Tel1p (Savitsky et al., 1995; Keegan et al., 1996). Atm and Atr are DNA damage inducible protein kinases that phosphorylate p53 to cause cell cycle arrest (Tibbetts et al., 1999; Waterman et al., 1998; Siliciano et al., 1997). Like Mec1p, Atm is

also important for the transcriptional activation of DNA damage inducible genes. NF-kappaB is a stress inducible transcription factor that is activated by Atm in response to DNA damage and protects cells from oxidative damage (Lee et al., 1998). Cells defective for Atm display hypersensitivity to ionizing radiation, higher levels of residual DSBs, sensitivity to oxidative stress, and growth retardation (Foray et al., 1997; Taylor et al., 1994; Pandita and Hittelman, 1992; Taylor et al, 1975). Cells deficient in Atr also display sensitivity to DNA damage, particularly UV irradiation, and cell cycle checkpoint failure following damage (Wright et al., 1998; Cliby et al., 1998). In addition to its role as a somatic cell cycle checkpoint, Atm is required for meiosis. Atm, and Mec1p in yeast, are required for a DSB checkpoint that functions as a normal part of meiotic recombination (Pandita et al., 1999; Barlow et al., 1998; Flaggs et al, 1997; Lydall et al., 1996; Keegan et al., 1996; Savitsky et al., 1995) A-T patients display infertility and *ATM-/-* mice are completely sterile (Xu et al., 1996; Barlow et al., 1996). Thus *ATM*, and the related gene *ATR*, are critical components of DNA repair machinery that are required for genome stability, radioresistance, organismal development, and meiosis.

CURRENT FINDINGS

Telomeric heterochromatin requires numerous proteins and is modified by many others. To identify other factors that could modify or regulate telomeric silencing, a screen was performed for genes and sequences that would disrupt silencing when present in high copy number. Previously known silencing factors Sir3p and Sir4p were shown to disrupt

silencing when their dosage was increased. In contrast, increased dosage of Sir2p improved silencing at telomeres, as well as *HM* loci and the rDNA. These findings suggest Sir2p is limiting for silencing throughout the genome and that Sir3p and Sir4p are normally required in specific stoichiometries. In addition to their roles in silencing, *SIR2*, *SIR3*, and *SIR4* have been implicated in NHEJ. Cells deficient for any one of these genes are severely compromised in end-joining, based on plasmid repair assays. *SIR* mutant strains are as crippled in NHEJ as strains deficient for *HDF1* or *HDF2* (Mills et al., 1999; Tsukamoto et al., 1997). Deletion of *SIR2*, *SIR3*, or *SIR4* results in derepression of *HM* loci and simultaneous expression of **a** and α mating type information. Cells that express both **a** and α mating type information lose mating proficiency, but display an elevated efficiency of *RAD52*-dependent homologous recombination. One report has indicated that the NHEJ defect in *SIR* mutant strains was also due to the mating type effect (Astrom et al, 1999). However, telomeric heterochromatin factors respond to DSBs, suggesting a direct role in NHEJ (Mills et al., 1999; Martin et al., 1999).

Because the Sir complex is required for NHEJ, the role of Sir3p was investigated (Chapter 3). Sir3p responds to DSBs by redistributing from telomeres to sites of DNA damage in a process that requires the *MEC1*-dependent cell cycle checkpoint (Mills et al., 1999; Martin et al., 1999).

Another gene identified in the telomere desilencing screen was the anti-silencing factor gene *ASF1* (Le, et al., 1997). *ASF1* encodes a small, highly acidic protein whose expression oscillates during the cell cycle, reaching peak levels during S-phase. Mutation of *ASF1* results in sensitivity to a variety of DSB inducing agents and an inability to perform NHEJ.

Asf1p is conserved from yeast to human and represents a new DNA repair factor that links heterochromatin formation to DSB repair. The role of *ASF1* in NHEJ is discussed in Chapter 4. These findings illuminate the role of silencing factors in DSB repair in yeast and highlight the importance of telomeres in regulating the availability of DNA repair factors to the rest of the genome.

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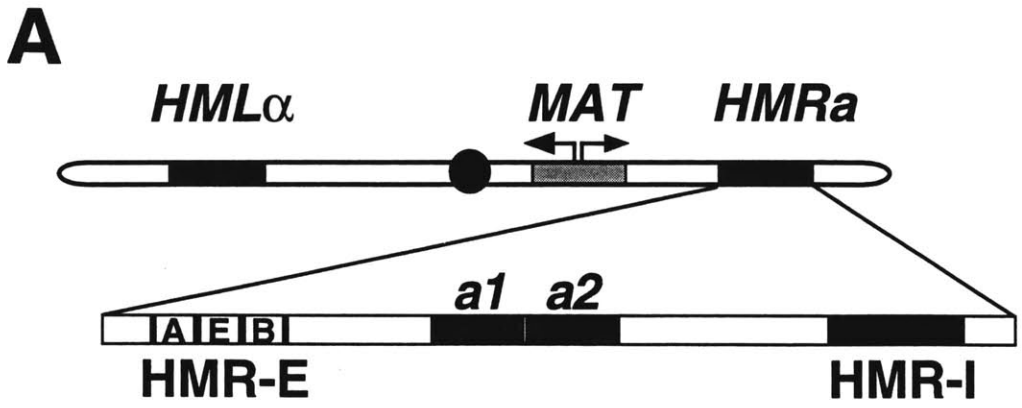
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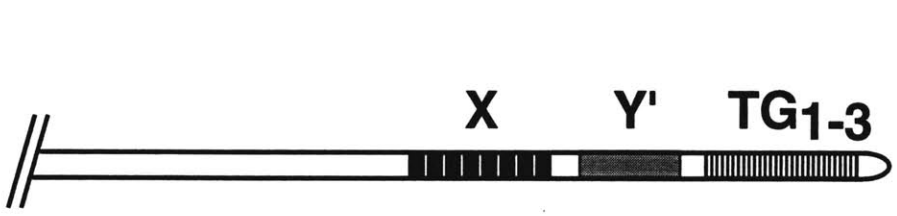
HMR-E
HMR-I


B
X
Y'
TG1-3

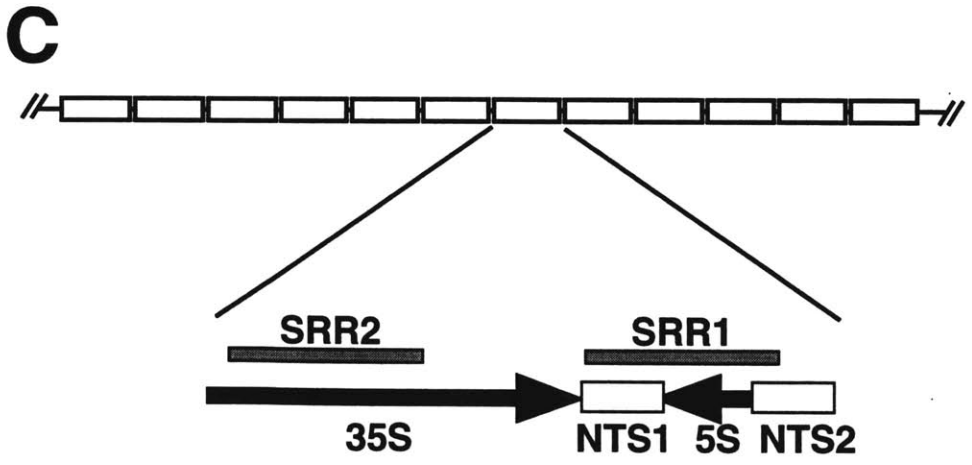

C
SRR2
SRR1
35S
NTS1
5S
NTS2

FIGURE 1- Schematic representation of silent loci in *Saccharomyces cerevisiae*. (A) Chromosome III containing *HMLα*, *MAT*, and *HMR***a**. Close up of the structure of *HMR* is shown. **a1** and **a2** are tge maring type **a** specific genes and HMR-E and HMR-I are the silencers. (B) Structure of a typical telomere containing terminal $TG_{1\text{-}3}$ sequence, a Y' element, and an X element. (C) Schematic of rDNA. The features of a single 9 kb repeat are shown in close up. SRR1 and SRR2 are the Sir2p-responsive regions and NTS1 and NTS2 are the non-transcribed spacer regions between the 35S and 5S rRNA genes.

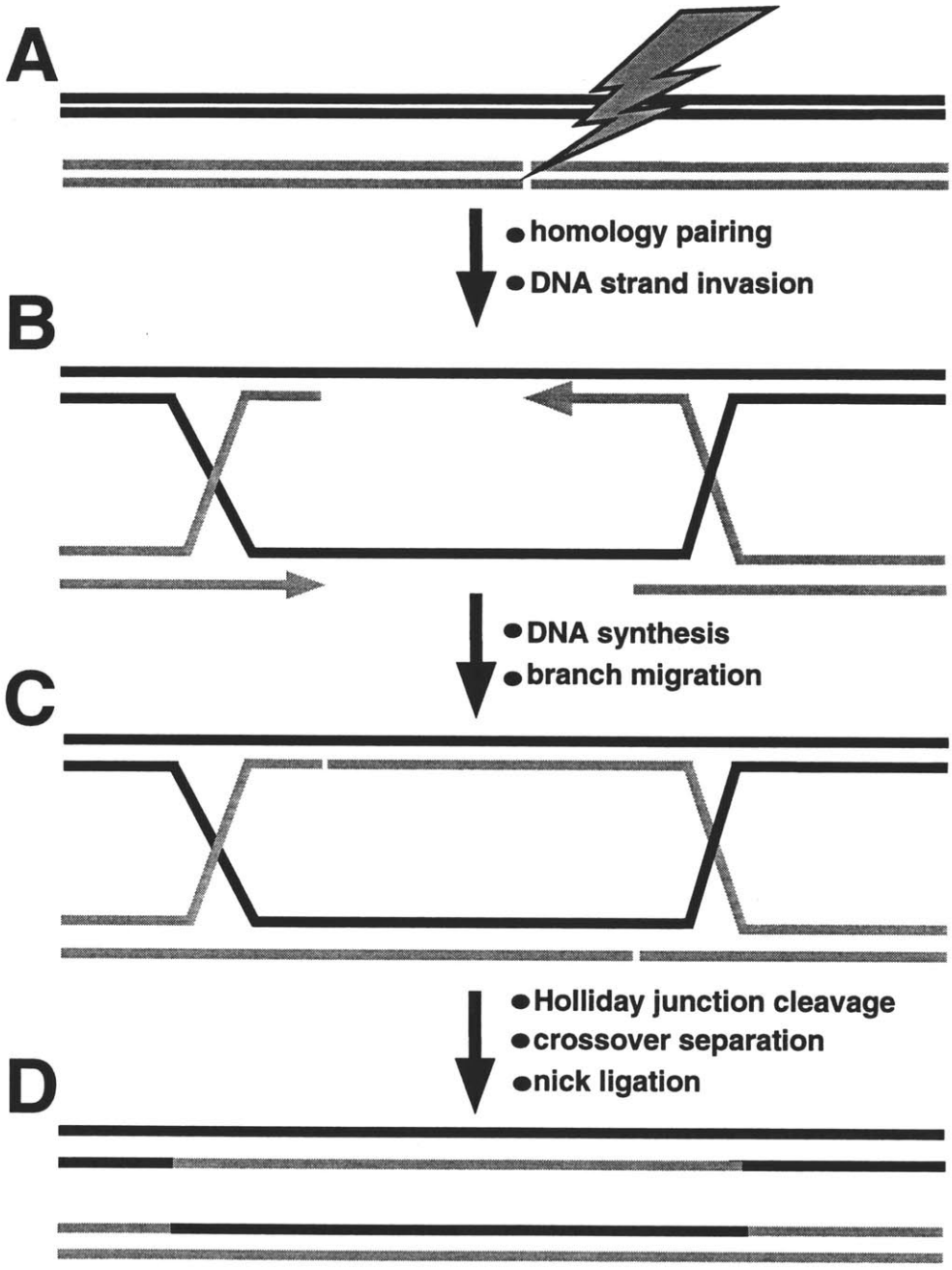
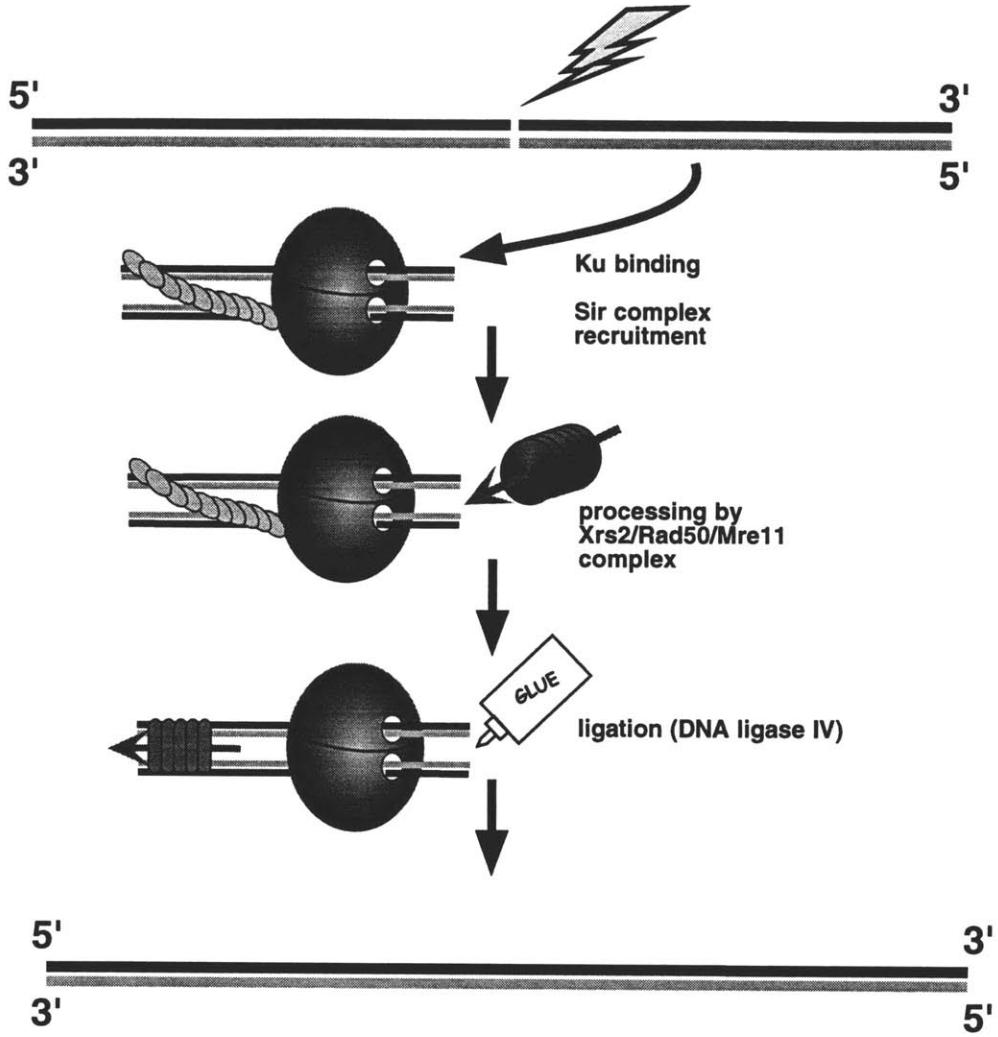

A
homology pairing
DNA strand invasion
B
DNA synthesis
branch migration
C
Holliday junction cleavage
crossover separation
nick ligation
D

FIGURE 2- Schematic of general steps of DSB repair by homologous recombination. (A) Induction of a DSB is followed by homology pairing and invasion of the broken strand into the intact DNS duplex. (B) Missing DNA is filled in by DNA synthesis and associated migration of the Holliday junction. (C) Two complete DNA duplexes joined by crossover junctions are separated by cleavage and separations of the crossovers. The nicks are sealed by ligation. (D) Two intact DNA duplexes.


5'
3'
3'
5'
Ku binding
Sir complex
recruitment
processing by
Xrs2/Rad50/Mre11
complex
GLUE
ligation (DNA ligase IV)
5'
3'
3'
5'

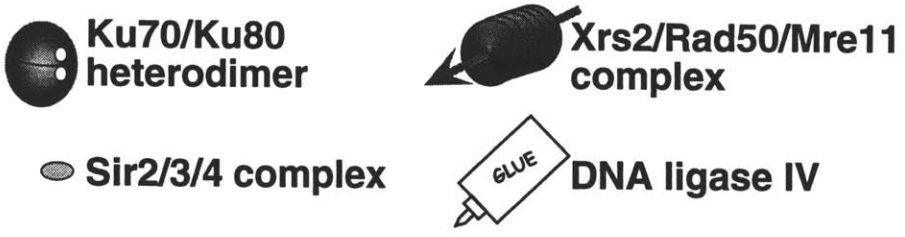

Ku70/Ku80
heterodimer
Xrs2/Rad50/Mre11
complex
Sir2/3/4 complex
GLUE
DNA ligase IV

FIGURE 3- Model of steps in nonhomologous end-joining in *Saccharmyces cerevisiae.* Induction of DSB is followed by binding of the Ku70/Ku80 heterodimer to DNA ends, recruitment of Sir complex to the DSB, processing of the by Xrs2p/Rad50p/Mre11p complex, and ligation of the break by DNA ligase IV.

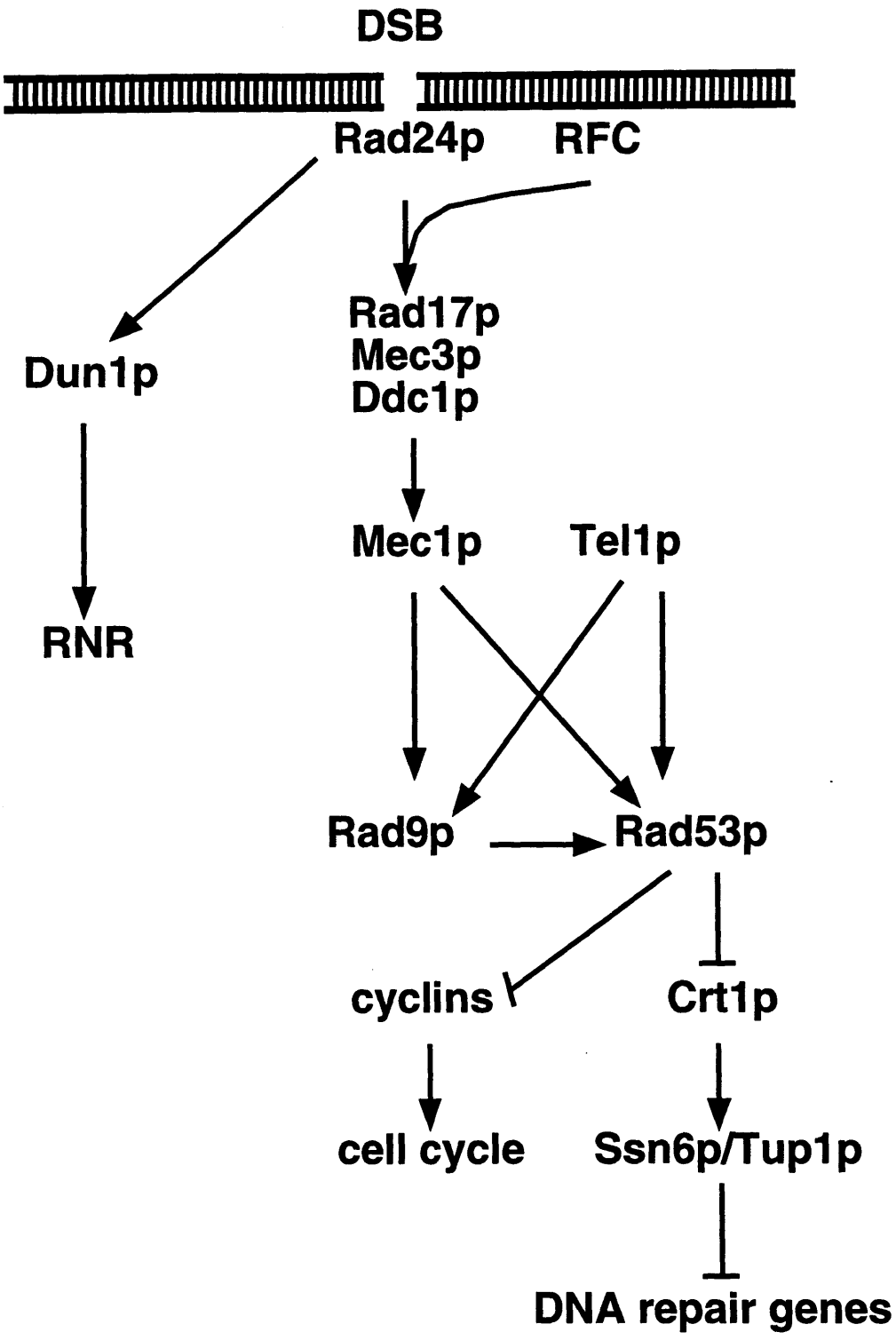

DSB
Rad24p
RFC
Dun1p
Rad17p
Mec3p
Ddc1p
RNR
Mec1p
Tel1p
Rad9p
Rad53p
cyclins
Crt1p
cell cycle
Ssn6p/Tup1p
DNA repair genes

FIGURE 4- Schematic representation of the S-phase DNA damage checkpoint *Saccharomyces cerevisiae*. Rad24p and RFC detect DNA damage and signal Rad17p, Mec3p, and Ddc1p which transduce the signal to the Mec1p kinase. Mec1p and Tel1p kinases can phosphorylate Rad9p and Rad53p, which interact to activate DNA repair genes and halt cell cycle progression. Detection of DNA damage also activates Dun1p, which activates ribonucleotide reductase.

Chapter 2: Identification and characterization of high-copy antagonists of silencing in *Saccharomyces cerevisiae*

The work presented in this chapter was performed in collaboration with David Sinclair.

Summary

In *Saccharomyces cerevisiae* position dependent gene repression, known as silencing, is associated with the cryptic mating-type loci, telomeres, and the rDNA. To identify additional modifiers of position effect we screened a high copy yeast genomic library for genes that could derepress a silent telomeric marker. Telomere desilencing genes were characterized for their effects on *HM* and rDNA silencing. The genes identified in this screen include *MAT***a**, the antisilencing factor *ASF1*, *SIR3* and *SIR4*, and *SAS2*. High copy *SIR2* enhanced silencing at all loci tested. These results may indicate that silencing is a carefully regulatable process and potentially identify Sir2p as a limiting factor for silent chromatin.

Introduction

Position dependent regulation of gene expression is a common theme in eukaryotes. In Drosophila this phenomenon is known as position effect variegation and results in variable expression of genes located near boundaries between transcriptionally active euchromatin and transcriptionally inert heterochromatin (reviewed in Eissenberg 1989; Henikoff 1992). In *Saccharomyces cerevisiae* position dependent repression, known as silencing, is associated with the silent mating type loci, telomeres, and rDNA. Marker genes placed near the silent mating type loci (*HMR***a** and *HML*a) are efficiently silenced (Schnell and Rine 1986; Mahoney and Broach 1989) whereas marker genes placed near a telomere are stably but reversibly silenced (Gottschling et al. 1990; Renauld et al. 1993). More recently silencing was shown to be associated with the tandemly repeated rDNA array (Bryk et al. 1997; Smith and Boeke 1997). Transcriptional silencing of genes placed within the rDNA appears to be weaker than that associated with telomeres and *HM* loci.

Silencing is dependent on several proteins. The Silent Information Regulators (*SIR1-4*) are all required for stable and efficient silencing at the *HM* loci (Ivy et al. 1986; Rine and Herskowitz 1987). Telomere silencing requires *SIR2*, *SIR3,* and *SIR4* but not *SIR1* (Aparicio et al. 1991). Silencing in the rDNA array is dependent on *SIR2* while *SIR3* and *SIR4* may play accessory roles (Bryk et al. 1997; Smith and Boeke 1997). Several other factors participate in silencing at *HM* and telomeres including the Repressor Activator Protein 1 (*RAP1*) (Shore and Nasmyth 1987; Kurtz and Shore 1991; Sussel and Shore 1991; Kyrion et al. 1993), the Origin Recognition Complex (ORC) (Bell et al. 1993), Histones H3 and H4

(Kayne et al. 1988; Johnson et al. 1990; Thompson et al. 1994; Hecht et al. 1995), and the ARS Binding Factor 1 (*ABF1*) (Shore et al. 1987; Buchman et al. 1988; Loo et al. 1995)

Sir2p, Sir3p, Sir4p, and Rap1p have been shown by several criteria to physically interact with one another (Palladino et al. 1993; Moretti et al. 1994; Cockell et al. 1995; Hecht et al. 1996; Gotta et al. 1997; Strahl-Bolsinger et al. 1997; Gotta et al. 1998). In addition, Sir3p, Sir4p, Rap1p, and to a limited extent Sir2p, colocalize with telomeric DNA in discrete foci associated with the nuclear periphery (Palladino et al. 1993; Gotta et al. 1997). Additionally Sir3p and Sir4p interact with Histones H3 and H4 (Johnson et al. 1990; Hecht et al. 1995). These observations suggest that silencing is mediated by large, multi-protein complexes. The molecular nature of such complexes is, as yet, poorly understood.

In addition to the many protein factors involved in silencing, several *cis*-acting elements that participate in silencing have been identified. Each of the silent mating type loci is flanked by DNA elements termed silencers (Nasmyth and Tatchell 1980; Strathern et al. 1980; Abraham et al. 1984; Feldman et al. 1984; Brand et al. 1985). The best characterized of these silencers is the *HMR*-E silencer. This element consists of three sub-domains: the A region, which contains an ARS consensus sequence bound by the Origin Recognition Complex; the E region bound by Rap1p; and the B region bound by Abf1p (Brand et al. 1985; McNally and Rine 1991; Bell et al. 1993). This silencer contains some redundancy since mutation of any single sub-domain results in, at most, a partial loss of silencer function. However, mutation of any two sub-domains is sufficient to completely abolish silencer function (Brand et al. 1987). Sequence elements that participate in telomere silencing have also been identified. The terminal

sequences at yeast telomeres are composed of a repeated $C_{1-3}A$ sequence that contains many binding sites for Rap1p (Shampay et al. 1984; Walmsley et al. 1984; Longtine et al. 1989; Conrad et al. 1990, Marcand et al. 1997). The precise sequences responsible for mediating rDNA silencing have not been identified but Sir2p appears to preferentially associate with the non-transcribed spacer (NTS) region between the divergently transcribed 35S and 5S rRNA genes (Fritze et al. 1997; Gotta et al. 1997).

To identify protein factors that participate in silencing complexes and to identify additional *cis*-acting sequences that may be the targets for silencing complexes a screen for derepression of a transcriptionally silenced telomeric marker was performed. Nine genes were identified which, when present in high copy number, desilence the telomeric marker. These included *SIR3*, *SIR4*, *ASF1* (Le et al. 1997; Singer et al. 1998), *SAS2* (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997), and *NPL3* (Bossie et al. 1992; Flach et al. 1994; Loo et al. 1995; Lee et al. 1996), all previously shown to affect silencing, as well as the transcriptional regulator gene *SPP41* (Maddock et al. 1994). In addition the silent mating type loci, *HMLa* and *HMR***a**, and the expressed *MAT***a** locus were isolated in this screen. The effect of each of these high copy number telomere desilencers on *HM* and rDNA silencing was also tested.

RESULTS

Screen for antagonists of telomere silencing

A high-copy screen for derepression of a silenced telomeric marker was performed. For this purpose a strain was constructed that contained

ADE2 near telomere V-R and *URA3* near telomere VII-L (PSY316AUT). Silencing of the telomeric *ADE2* marker results in formation of a red colony while expression of the *ADE2* gene results in formation of a white colony. This strain normally exhibits silencing in approximately 50% of the cells in a population, resulting in a variegated red-white phenotype thus providing a sensitive color assay for perturbation of telomeric silencing. By identifying colonies that were substantially less red than wild-type a high copy number (2μ) genomic library was screened for plasmids which weakened or eliminated telomere silencing. Candidates were then scored for ability to grow in the absence of supplemented uridine. Growth was monitored by microscopically examining cells plated to synthetic complete medium lacking uridine after an overnight incubation at 30^{o}. Strains that resulted in growth from greater than 50% of the cells plated were chosen for further analysis. The screen protocol is diagrammed in Figure 1.

A total of 80,000 colonies were screened yielding 28 plasmids that caused a loss of silencing of both telomeric marker genes. Sequencing across the vector-insert junctions showed that the desilencing plasmids constituted nine groups. Because the average insert size was 10 kb it was necessary to identify the region on each plasmid responsible for the silencing phenotype. For plasmids that showed less than approximately 10% red-sectored colonies, transposon mutagenesis was employed. At least two plasmids from each group were mutagenized with a modified Tn*1000* transposon (Morgan et al. 1996) to generate pools of randomly distributed Tn*1000* insertions. These pools were then screened for the loss of high copy desilencing activity using the *ADE2* assay by identifying colonies in which red color was restored. Mutagenized plasmids were recovered and

sequenced across the insertion boundary to identify the disrupted region. The results of the transposon mutagenesis are summarized in Figure 2. Two plasmid groups resulted in weaker desilencing than the rest. However, these plasmids carried either *SAS2* or *NPL3,* each of which had previously been implicated in silencing (Loo et al. 1995; Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). *SAS2* and *NPL3* were subcloned into a high copy vector and tested for desilencing activity. The subcloned genes behaved identically to the genomic library plasmids (data not shown) and were used for all subsequent experiments. In total, seven genes were identified which, when present in high copy number, result in loss of telomere silencing. In addition, plasmids containing *HMLa* and *HMR***a** were capable of derepressing the silenced telomeric markers (Table 3).

Telomere silencing assays

Plasmids identified in this screen were characterized for telomere silencing using the color based *ADE2* assay and a quantitative 5-fluoroorotic acid sensitivity assay. Both *SIR3* and *SIR4* resulted in strong telomere desilencing (Figure 3), but *SIR2* was not isolated in this screen. We wished to determine if high copy *SIR2* would behave similarly to *SIR3* and *SIR4*. A 2μ plasmid containing *SIR2* was introduced into the *ADE2 URA3* telomere reporter strain. In contrast to *SIR3* and *SIR4* high copy *SIR2* caused a strong increase in telomere silencing by the color assay (Figure 3). High copy *MAT***a**, *ASF1*, and *SPP41* also exhibited a strong effect on silencing of telomeric *ADE2*. High copy *SAS2*, *NPL3*, *HMR***a**, and *HMLa* also resulted in derepression of *ADE2,* though not as strongly as *SIR3* or *SIR4*. To determine the relative strength of the desilencing conferred by each plasmid the percent of red or sectored colonies produced

by each was determined by plating to sellective medium containing low adenine and scoring red, white, and sectoring colonies after 3-4 days (Table 3).

To obtain a more quantitative measure of the effect of each plasmid on telomere silencing, repression of the telomeric *URA3* marker was examined. Expression of *URA3* results in sensitivity to the drug 5-fluoroorotic acid. Repression of *URA3* confers 5-FOA resistance and provides another sensitive assay of the fraction of the cells in a population in the silenced state. Consistent with the color based assay *SIR3*, *SIR4*, *ASF1*, *SPP41*, *MAT***a**, and *HML*α all resulted in a 10-100 fold increase in 5-FOA sensitivity while *SIR2, SAS2*, *NPL3*, and *HMR***a** had no detectable effect on 5-FOA sensitivity (Figure 4).

To verify that the plasmids isolated in this screen indeed modified telomere position effect, their effects on expression of *ADE2* located at its native locus were examined. A wild-type copy of *ADE2* was integrated at the native *ADE2* locus in PSY316 thereby correcting the *ade2-101* allele. High copy telomere desilencing plasmids were introduced and the resulting strains were patched onto selective medium containing low adenine to assay color (Figure 5A). None of the plasmids resulted in red color formation, indicating that *ADE2* was not repressed by any of the telomere desilencers. Notably, *SIR2*, which results in increased red colony formation when *ADE2* is located near a telomere, had no effect on color when *ADE2* was located at its native locus. To further establish that the telomere desilencing plasmids had no effect on expression of *ADE2* at at a non-silenced locus, Northern blot analysis was performed. As expected, presence of high copy desilencers had no significant effect on the level of *ADE2* mRNA when expressed from its native locus (Figure 5B). These

results indicate that the plasmids isolated in this screen contain *bona fide* high copy antagonists of telomere position effect.

Effects of telomere desilencers on *HMR* silencing

To test the effects of each high copy plasmid on silencing of *HMR*, a reporter strain bearing *HMR::ADE2* integrated at *HIS3*, and *TRP1* integrated at the native *HMR* locus was used. To test for a loss of silencing, expression of *ADE2* was tested by a color assay on low adenine medium. A low level *ADE2* expression in this strain results in formation of red colonies for cells carrying vector alone. Overexpression of *SIR3*, *SIR4*, *ASF1*, *HMR***a** and *HML*α strongly decreased silencing; *SIR2*, *SAS2*, *MATa*, *NPL3*, and *SPP41* had no apparent effect (Figure 6).

Effects of telomere desilencers on rDNA silencing

In addition to telomeres and *HM* loci, silencing has recently been identified in the rDNA array. In otherwise wild-type cells rDNA silencing absolutely requires *SIR2* but not *SIR3* or *SIR4* (Bryk et al. 1997; Smith and Boeke 1997). Other participants in rDNA silencing have not been characterized. To determine their effects on rDNA silencing, each plasmid was introduced into a strain that contains the *ADE2* gene integrated into the rDNA array. Silencing of *ADE2* results in a decreased fraction of cells that can grow without supplemented adenine. *SPP41* had little effect on rDNA silencing, but all other genes isolated in this screen resulted in desilencing of the rDNA *ADE2* marker (Figure 7). *ASF1* had a notably strong effect. Again *SIR2* had the opposite effect of *SIR3* and *SIR4* at the rDNA, resulting in at least 10-fold stronger silencing of *ADE2*.

ASF1

ASF1 was previously identified as a high copy desilencer of *HML* (Le et al. 1997). High copy number *ASF1* also results in strong derepression of a marker at telomeres or the rDNA. One possibility suggested by these data was that *ASF1* encoded an "antisilencer" protein, whose function was to counteract silencing. To further define the role of *ASF1* in telomere silencing, the ORF was disrupted in PSY316AT. Two independent disruption isolates were tested for telomere silencing using the color-based *ADE2* assay. Disruption of *ASF1* resulted in a dramatic increase in telomere silencing as well as a slight slow growth phenotype (Figure 8).

Although overexpression of *ASF1* affected silencing at all tested loci, it was still possible that Asf1p was a locus specific silencing factor. To determine the role of *ASF1* in silencing at the rDNA, it was disrupted in W303AR. Again, two independent isolates were tested. Although *ASF1* disruption strongly influenced silencing at telomeres, there was no detectable change in the strength of rDNA silencing in a W303AR *ASF1*-disrupted strain (Figure 9). These results suggest that *ASF1* is not a global regulator of silencing but may negatively regulate silencing in specific nuclear compartments.

DISCUSSION

We screened a yeast genomic library for plasmids which, when present in high copy number, compromise telomere silencing. Similar screens for *HM* or telomeric desilencing have yielded modifiers of position effect such as *ASF1*, and participants in telomere maintenance such as *TLC1* (Singer and Gottschling 1994; Singer et al. 1998). This screen was

anticipated to yield three classes of silencing effectors: DNA sequence elements bound by silencing factors, components of silencing complexes, and antisilencing factors. The results of this screen are summarized in Table 4.

Silencing requires both protein factors and DNA sequence elements that are bound by protein factors. At telomeres the repeated $C_{1-3}A$ terminal tracts contain multiple Rap1p binding sites and the silent mating type loci are flanked by a complex array of silencer elements. Because silencing-associated DNA elements are bound by protein factors that play a central role in silencing, it was reasoned that introduction of multiple copy silencer elements from the 2μ genomic library would compromise telomere silencing by recruiting protein factors to the plasmid-borne silencers. Indeed, both *HMLα* and *HMR***a** were identified as high copy telomere desilencers.

ASF1

ASF1 encodes a small, highly acidic protein. *ASF1* was originally identified in a high copy screen for derepression of an *hml::TRP1* reporter and resulted in a loss of silencing at *HM* loci and telomeres when overexpressed (Le et al. 1997; Singer et al. 1998). Expression of *ASF1* increased during the S-phase of the cells cycle and an *asf1* mutant strain displayed increased sensitivity to the DNA alkylating agent methyl methane sulfonate (MMS) and to ionizing radiation (Le et al. 1997).

In our screen for high copy telomere desilencers *ASF1* was isolated three times (Table 3). In addition to telomeres, we examined the effect of *ASF1* overexpression on silencing at *HMR* and at the rDNA. Overexpression of *ASF1* decreases the strength of silencing at all three loci

assayed. The role of *ASF1* in silencing was further characterized by assaying telomere or rDNA silencing in strains disrupted for *ASF1*. Disruption of *ASF1* resulted in enhanced telomere silencing but had no effect on rDNA silencing. These results suggest that Asf1p counteracts silencing, but that this activity is normally excluded from the rDNA. It is curious that overexpression, but not disruption of *ASF1* had an effect on rDNA silencing. It is possible that the rDNA is not a normal target of Asf1p, but overexpression results in an expanded or inappropriate range of chromosomal targets, perhaps through interactions with factors shared by telomeres and rDNA.

Asf1p could function as an antisilencer by activating the transcription of genes embedded in silent chromatin. Such a mechanism might be employed to prevent repression of genes located near telomeres or *HM* loci. Alternatively, Asf1p could act as an antagonist of chromatin formation. This model is supported by the observation that passage through S-phase is required for the establishment of silencing and *ASF1* mRNA levels increase during this phase of the cell cycle (Miller and Nasmyth 1984; Laman et al. 1995; Le et al. 1997). At telomeres silent chromatin is assembled and spreads inward toward the centromere (Renauld et al. 1993; Hecht et al. 1996). A silencing antagonist may be employed to prevent too great a spread of silencing from any single telomere. A similar mechanism may prevent excess silencing near *HM* loci.

*SIR*s

Considerable data suggest that Sir3p and Sir4p participate in silencing complexes and that altering the balance of these proteins alters the

efficiency of silencing. It has previously been shown that overexpression of either full length Sir4p or the C-terminal region of Sir4p interferes with silencing and alters normal localization (Marshall et al. 1987; Cockell 1995). A study characterizing the effects of Sir3p dosage demonstrated that overexpression of the Sir3p N-terminus could enhance telomere silencing and suppress the desilencing caused by Sir4p overexpression (Gotta et al. 1998). Two-hybrid and immunoprecipitation experiments have shown that Sir4p interacts with Sir3p and with Rap1p (Palladino et al. 1993; Moretti et al. 1994; Cockell et al. 1995; Gotta et al. 1997). Overexpression of Sir3p or Sir4p may compromise silencing by inappropriately occupying interacting factors and interfering with normal complex formation or function.

Recently, overexpression of *SIR2* was shown to result in increased rDNA silencing, and Sir2p appears to be associated with specific sequences in the nontranscribed spacer region between the 5S and 25S rRNA genes (Fritze et al. 1997). The authors concluded that Sir2p was a limiting factor for silencing within the rDNA (Fritze et al. 1997). Here we show that Sir2p overexpression strongly enhances silencing at telomeres, *HMR*, and rDNA. This is consistent with the idea that Sir2p is the limiting factor for silencing. Because *SIR2* is essential for silencing at all known silent loci, a limited pool of Sir2p may result in competition between telomeres, *HM* loci (Buck and Shore 1995), and rDNA. Increasing the available Sir2p may alleviate this competition and result in elevated silencing at all loci.

It is interesting that multiple copy *SIR3* results in loss of telomere silencing in this system, but has been previously shown to increase the distance from a telomere over which silencing can act. Specifically, high copy number *SIR3* could extend silencing to approximately 25 kb from the

telomere (Renauld et al. 1993). Similarly, overexpression of the N-terminus of Sir3p enhances telomere silencing, in contrast to the derepressing effect of overexpression of full length Sir3p (Gotta *et al.*, 1998). The difference between these observations and the results presented in this report may be due to variability of limiting factors between strains, possibly interacting with the Sir3p N-terminus.

SAS2

SAS2 encodes a protein with homology to histone acetyltransferases. Mutation of *SAS2* enhances the silencing defect in a *sir1* mutant strain and causes a loss of telomere silencing, but can suppress the silencing defect of an *HMR*-E silencer with mutant Rap1p and Abf1p binding sites (Reifsnyder et al. 1996; Ehrenhofer-Murray et al. 1997). These observations suggest that Sas2p is important for modulating *HM* silencing, perhaps by balancing the distribution or activity of other silencing proteins between the four *HM* associated silencers. It has also been suggested that *SAS2* may either positively or negatively regulate silencing depending on the chromosomal context (Reifsnyder et al. 1996). In this study, overexpression of *SAS2* derepressed genes in a telomeric or rDNA, possibly mimicking the phenotypes of a *SAS2* truncation (Reifsnyder et al. 1996). This may indicate that Sas2p functions in a multi-protein complex, and that overexpression, like mutation, can disrupt the complex.

Other desilencing genes

In addition to *HML* and *HMR*, the expressed *MAT***a** locus conferred a strong loss of telomere silencing when present in high copy number. The *MAT***a** locus is identical to that at *HMR***a** but is not flanked by silencers

(Astell et al. 1981). In diploid cells the **a**1 gene product heterodimerizes with the α2 gene product to form an activator of diploid specific genes (reviewed in Herskowitz 1989). Additionally the **a**1-α2 transcription factor represses the α-gene specific activator α1. In mating type **a** strains, neither **a**1 nor **a**2 have any known function. In fact, no function has yet been demonstrated for **a**2 under any circumstances. In the Y**a** region, which contains part of the promoter between the divergently transcribed **a**1 and **a**2 open reading frames, is an Abf1p binding site (McBroom and Sadowski 1995). Presence of this site in multiple copies was initially thought to titrate silencing factors and compromise silencing. However, transposon mutagenesis of the 2μ plasmid containing *MAT***a** revealed that disruption of either the **a**1 or **a**2 ORF was sufficient to abrogate the high copy desilencing phenotype associated with *MAT***a**, suggesting that concurrent expression of **a**1 and **a**2 is responsible for telomere desilencing.

The product encoded by the *NPL3* gene has been implicated in export of RNA from the nucleus (Singleton et al. 1995; Bossie et al. 1992) and import of protein into the nucleus (Flach et al. 1994). A temperature sensitive mutation in *NPL3*, called *npl3-95*, was isolated in a sensitized screen for genes that affect *HM* silencing (Loo et al. 1995). At the permissive temperature *npl3-95* had a nuclear import defect and caused derepression of impaired or synthetic *HMR*-E silencers (Loo et al. 1995). *NPL3* was isolated in our screen as a high-copy number inhibitor of telomere silencing but had little, if any, effect on silencing at *HM* loci or the rDNA. The role of Npl3p in silencing is unclear, but it has been hypothesized that the silencing defect observed in a *npl3-95* strain is a result of aberrant nuclear localization of silencing effectors at the permissive temperature(Loo et al. 1995). Overexpression of *NPL3* may

result in loss of telomere silencing by similarly interfering with proper nuclear localization. Consistent with this idea is the observation that *NPL3* disruption results in neither a nuclear localization defect (Bossie et al. 1992), nor a silencing defect (Loo et al. 1995).

The essential gene *SPP41* was first identified as a suppressor of *prp4* temperature sensitive mutants (Maddock et al. 1994). *SPP41* encodes a negative transcriptional regulator of RNA processing genes (Maddock et al. 1994). When present in high copy number *SPP41* has a derepressing effect on telomeres and rDNA, but not on a reporter gene located at a non-silenced locus. This demonstrates that *SPP41* is a newly identified modifier of position effect. The nature of it activity is unclear, but may be caused by occupying interacting factors that are also neccessary for proper silencing.

The screen reported here identified seven genes that compromise telomere silencing when present in high copy number, including *MAT***a**, the antisilencer gene *ASF1,* members of the Sir complex, and the putative histone acetyltransferase gene *SAS2*. Additionally, plasmids containing the *cis*-acting elements associated with the cryptic mating type loci were isolated. These telomere desilencing plasmids were also tested for silencing phenotypes at *HM* loci and the rDNA. Interestingly a similar screen was recently reported that identified a largely non-overlapping set of genes (Singer et al. 1998). Taken together with our results this strengthens the notion that position effect in Saccharomyces is very sensitive to the dosage of many key components, including Asf1p, Sir3p, Sir4p, and Sas2p, and that Sir2p may be a limiting factor.

MATERIALS AND METHODS

Yeast strains and plasmids

Strains used in this study are listed in Table 1. The strains used for all telomere silencing assays in this study, PSY316AT and PSY316AUT, were generated by integrating the *ADE2* gene at TEL V-R (AT) or the *ADE2* gene at TEL V-R and the *URA3* gene at TEL VII-L (AUT) of PSY316. *ADE2* was integrated using the plasmid pHR10-6 (Singer and Gottschling 1994) and *URA3* was integrated using plasmid pVII-L URA3-TEL (Gottschling et al. 1990). *HM* silencing assays were carried out in YLS227 (Brand et al. 1987) containing *TRP1* silenced by an *HMR*-E silencer mutant for its Rap1p binding site. The rDNA silencing reporter strain was constructed by integrating plasmid pDS40 (gift of D. Sinclair, MIT), linearized with *Kpn*I, into the rDNA array. The high copy (2μ) *SIR2* plasmid, pLP350 (gift of L. Pillus, University of Colorado), was used for all silencing assays. All yeast transformations were performed using the lithium acetate method (Geitz et al. 1992).

Silencing Screen

PSY316AUT was transformed with a high-copy YEp13 based yeast genomic library (ATCC #37323). Transformations were plated to synthetic glucose medium lacking leucine and containing 0.02% adenine (SC-leu/low ade). Use of low adenine medium facilitated the development of red color without compromising the growth of cells with a silent *ADE2*. Wild-type PSY316AUT grown on SC-leu/low ade medium forms sectored colonies. Library transformants were screened for loss of telomeric silencing by assaying formation of white colonies that no longer sector. These were then tested for desilencing of the telomeric *URA3* by scoring growth on synthetic medium lacking uridine (SC -ura). Library plasmids

were isolated from candidate transformants and re-introduced into PSY316AUT. Transformants were reassayed for desilencing of the *ADE2* marker by scoring colony color on SC-leu/low ade medium. Plasmids that retained desilencing activity after retransformation were sequenced across the vector-insert junction. Sequences were submitted for comparison to the Saccharomyces Genome Database using the Basic Local Alignment Search Tool (BLAST).

Silencing assays

The telomere silencing phenotype conferred by each plasmid was assayed by streaking PSY316AUT containing each plasmid to SC-leu/low ade medium. Cells were grown for 3-4 days at 30^{o} then placed at 4^{o} to enhance development of red color. To obtain a quantitative measure of the loss of silencing SC-leu liquid cultures of PSY316AUT harboring each plasmid were grown overnight at 30^{o}. Cultures were pelleted by centrifugation, washed once in sterile water, and resuspended in 1 ml sterile water. Cell density was measured by determining OD_{600} of the 1 ml suspension and was adjusted to OD_{600}=1. Ten-fold serial dilutions were made in sterile water and 10 ml each dilution was spotted onto SC-leu and SC-leu containing 5 mg/ml 5-fluoroorotic acid (SC-leu/5-FOA). SC-leu plates were incubated for 2 days at 30^{o}. SC-leu/5-FOA plates were incubated 2 or 3 days at 30^{o}.

HM and rDNA silencing assays were performed as described for assaying the telomeric *URA3* marker. Overnight cultures were washed in water, adjusted to OD_{600}=1, and spotted to SC medium lacking tryptophan (SC-trp) or SC medium lacking adenine (SC-ade) for the *HM* assay or SC-

ade for the rDNA assay. *HM* and rDNA spot assays were grown overnight at 30^{o}.

Transposon Mutagenesis

Transposon mutagenesis of each telomere desilencing plasmid was performed using a modified bacterial Tn*1000* transposon (gift of S. Sedgwick). Mutagenesis using Tn*1000* derivative MH1598 (referred to as Tn*HIS3*), containing yeast *HIS3*, was carried out as described (Morgan et al. 1996). After transposition and mating were carried out, the mating mixture was plated to LB agar plates containing 400 mg/ml each of ampicillin and carbenicillin. Resulting colonies, each containing a single YEp13 plasmid harboring a transposon, were harvested to generate a pool of plasmids containing random transposon insertions. PSY316AUT was transformed with each transposon-mutagenized pool and screened for restoration of red sectoring indicating a loss of desilencing activity from the mutagenized plasmid. Plasmids from red sectoring colonies were sequenced near the transposon insertion site to identify the disrupted region. Sequence flanking the transposon insertions on both sides was obtained using oligonucleotidesTn1000-γ and Tn1000-δ (Table 2) directed against the γ and δ repeats, respectively, present at the ends of Tn*1000.*

Subcloning

Transposon mutagenesis of the YEp13 plasmids containing *SAS2*, *NPL3*, *HMR***a**, and *HML*α was not carried out due to difficulty identifying restoration of the wild-type silencing phenotype. These candidate genes were subcloned into the high copy number (2μ) vector pDB20Leu-*Bgl*II. Oligonucleotides used for PCR reactions are listed in Table 2. The entire

SAS2 open reading frame, including 348 bp upstream of the initiation ATG was amplified by the polymerase chain reaction from YEp13 clone 61 using oligonucleotides SAS2 START and SAS2 END, both of which contain an engineered *Hind*III site. The *ADH1* promoter (P*ADH1*) was removed from the high copy number (2μ) vector pDB20Leu-*Bgl*II by digesting with *Hind*III and religating the 6120 bp product to restore the *Hind*III site. The *SAS2* PCR product was cloned into the restored *Hind*III site to generated plasmid pSAS-123. To verify that pSAS-123 behaved similarly to the original library plasmid, PSY316AUT was transformed with pSAS-123 and the color-based silencing assay was performed.

NPL3 was cloned into pDB20Leu-*Bgl*II digested with *Pst*I and *Bam*HI to remove the P*ADH1*. The *NPL3* open reading frame and the entire promoter were amplified by PCR from YEp13 clone 120 using oligonucleotides NPL3 START and NPL3 END. *ASF1* was also cloned into the pDB20Leu-*Bgl*II derivative used for *SAS2* subcloning. The entire *ASF1* open reading frame, including 1160 bp upstream of the initiation ATG, was amplified by PCR from YEp13 clone 119 using oligonucleotides ASF5'HSF and ASF 3' FNH2, each containing an engineered *Hind*III site. The *ASF1* PCR product was then cloned into the *Hind*III site of the modified pDB20Leu-*Bgl*II plasmid to generate pASF3. Desilencing activity was confirmed by transforming PSY316AUT with pASF3 and performing the color based silencing assay.

Northern Blot analysis

Northern blot analysis of the *ADE2* reporter gene was carried out for high copy desilencing plasmids in PSY316AUT or yCA1 (Table 1). Total RNA was isolated from 5 ml ovenight cultures of yeast grown in SC-

leu liquid medium. RNA was prepared by lysis at 65^{o} in equal volumes TES (10 mM Tris-HCl pH7.5, EDTA pH 8.0, 0.5% SDS) and acid phenol, followd by extraction once each with acid phenol and chloroform. RNA was precipitated with 2.7 volumes cold, absolute ethanol, pellets were washed once with 70% ethanol, and resuspended in 30 µl water containing the RNase inhibitor RNAsin (Promega). Approximately 20 µg total RNA was loaded onto a 1% agarose gel containing 2% formaldehyde and electrophoresed at 50-100V. RNA was transferred to nylon membrane and probed with a ^{32}P-radiolabelled 900 bp *ADE2* probe. *ACT1* probe was used as a loading control.

Gene disruption

ASF1 was disrupted using the Tn*HIS3* (MH1598) mutagenized *ASF1* containing YEp13 library plasmid. *ASF1* plasmids containing a transposon insertion that interrupted the open reading frame of *ASF1* were identified by phenotype and confirmed by sequencing using primers Tn1000-g and Tn1000-d. One such mutagenized plasmid, called 119t-2 (Figure 2), was used for all gene disruptions. Plasmid 119t-2 was digested with *Sna*BI and *Stu*I. An approximately 6 kb fragment, containing the *HIS3* modified Tn*1000* sequence flanked by *ASF1* sequence, was gel purified and introduced into yeast. Disruptants were selected by growth of medium lacking histidine and confirmed by Southern blot and Western blot analysis.

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TABLE 1

Strains used in this study

Strains	Genotype	Source
PSY316	*MATα ura3-52 leu2-3,112 his3-Δ200 ade2-101 lys2-801*	Laboratory strain
W303-1A	*MAT***a** *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 can1-100*	R. Rothstein
ySB0163	W303-1A; *HMR::TRP1 his3::HMR::URADE2*	S. Bell
yCA1	PSY316; *ADE2*	C. Armstrong and L. Guarente
PSY316AT	PSY316; *ADE2*-TEL V-R	Laboratory strain
PSY316AUT	PSY316; *ADE2*-TEL V-R *URA3*-TEL VII-L	This study
W303AR	W303-1A; *ADE2*-rDNA	Sinclair and Guarente, 1998
KMY122	PSY316AT; *asf1::*Tn*1000-HIS3*	This study
KMY154	W303AR; *asf1::*Tn*1000-HIS3*	This study
KMY155	W303AR; *asf1::*Tn*1000-HIS3*	This study

TABLE 2

Oligonucleotides used in this study

Oligonucleotide	Sequence
Tn1000-γ	5'-CCTGAAAAGGGACCTTTGTATACTG-3'
Tn1000-δ	5'-AGGGGAACTGAGAGCTCTA-3'
SAS2 START	5'-GGGGGAAGCTTTTATATTTCTTAAGACACTC-3'
SAS2 END	5'-GGGGGAAGCTTTGCCATTAAGTTACATCCTG-3'
NPL3 START	5'-GGGGGCTGCAGTACCGCAGTATATTTTATAG-3'
NPL3 END	5'-GGGGGGGATCCACTCGCAATAAACAATTCTG –3'
ASF5'HSF	5'-GGGGGAAGCTTGAGCTCGGCCGGCCGATATCAACTACGAGAGCGATCG-3'
ASF 3' FNH2	5'-CCCCCCAAGCTTAGCGGCCGCGGCCGGCCGTTGAACG TGCCGCATCCTTTGG-3'

TABLE 3

Genes isolated as high-copy number telomere desilencers

Gene	Times isolated	Plasmid analysis	Percent red or sectored colonies
PSY316AUT			50
ASF1	3	transposon	0-5
SIR3	4	transposon	0-5
SIR4	5	transposon	0-5
*MAT***a**	7	transposon	0-5
SPP41	2	transposon	0-5
HMLα	3	subclone	10-20
*HMR***a**	1	subclone	10-20
SAS2	1	subclone	20-30
NPL3	2	subclone	20-30
SIR2		subclone	>90

YEp13 plasmids having desilencing activity were analysed by transposon mutagenesis or by individual cloning of candidate genes. Percent red or sectored colonies of PSY316AUT bearing high-copy number plasmids containing the indicated gene are shown. The high-copy plasmid pLP350, containing *SIR2*, was included for comparison.

TABLE 4

Silencing phenotypes of genes identified in telomere desilencing screen

Gene	*HM*	Telomere	rDNA
SIR2	N.A.	more	more
SIR3	less	less	less
SIR4	less	less	less
SAS2	N.A.	less	less
NPL3	no effect	less	less
SPP41	no effect	less	no effect
HMLα	less	less	less
*HMR***a**	less	less	less
*MAT***a**	less	less	less
ASF1	less	less	less
ASF1 disruption	N.A.	more	no effect

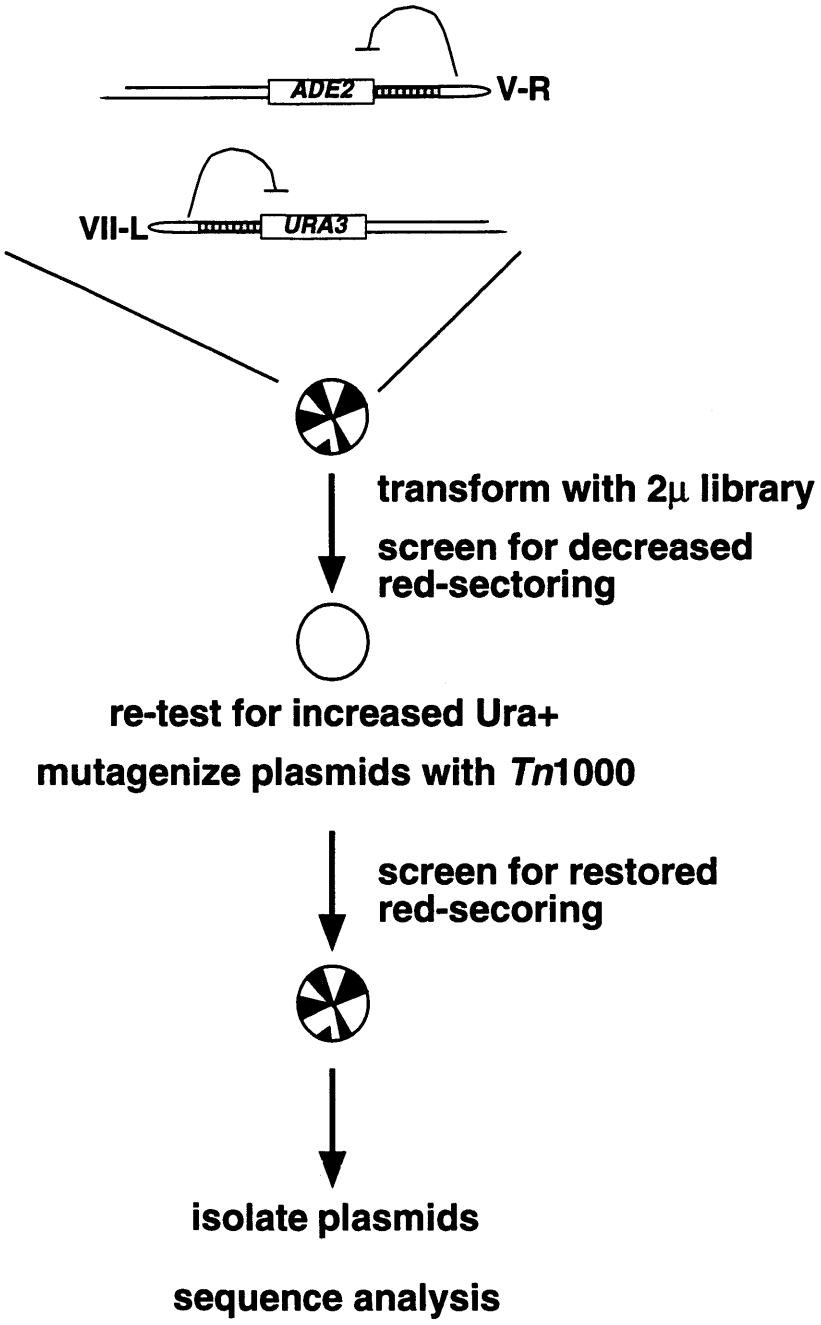

ADE2
V-R
VII-L
URA3
transform with 2μ library
screen for decreased red-sectoring
re-test for increased Ura+
mutagenize plasmids with Tn1000
screen for restored red-secoring
isolate plasmids
sequence analysis

FIGURE 1- Schematic representation of the telomere desilencing screen. A telomere silencing reporter containing *ADE2* at TEL V-R and *URA3* at TEL VII-L was constructed (PSY316AUT). Wild-type colonies of PSY316AUT develop red and white sectors. A yeast 2μ based genomic library was screened for high copy plasmids that derepress the telomeric markers resulting in loss of red sectors and increased sensitivity to 5-FOA.

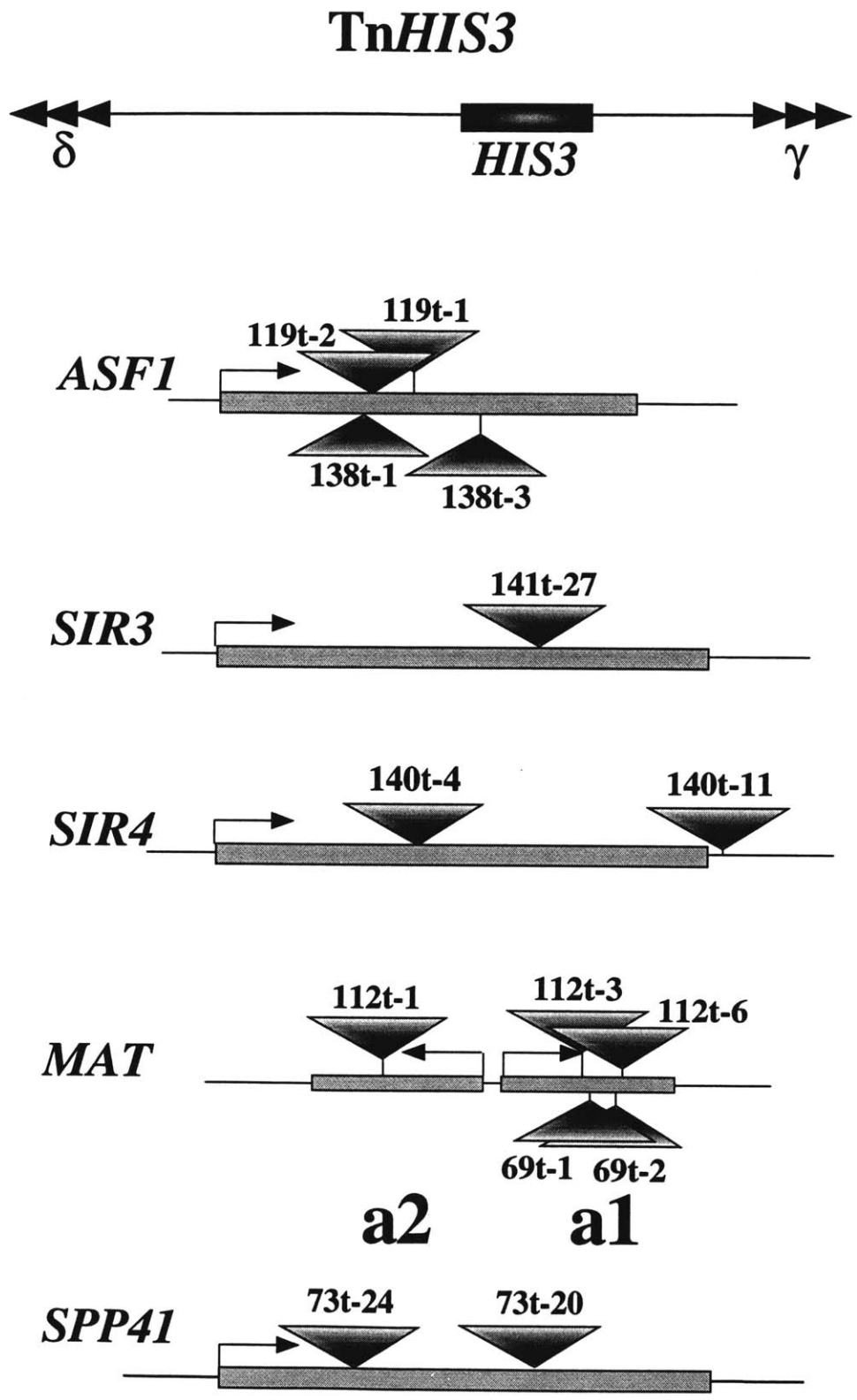

Tn*HIS3*
δ
HIS3
γ
ASF1
119t-1
119t-2
138t-1
138t-3
SIR3
141t-27
SIR4
140t-4
140t-11
MAT
112t-1
112t-3
112t-6
69t-1
69t-2
a2
a1
SPP41
73t-24
73t-20

FIGURE 2- Summary of Tn*HIS3* transposon mutagenesis of desilencer plasmids. *ASF1, SIR3, SIR4, MAT***a**, and*SPP41*-containing plasmids were mutagenized with a modified Tn*1000* containing *HIS3*. Tn*HIS3* transposon is shown with *HIS3* as a shaded box. The terminal d and g repeats are shown as arrowheads. Transposon insertions are depicted as shaded triangles and insertion sites relative to the open reading frame of each gene are shown. All transposon insertions shown resulted in a restoration of wild-type telomere silencing when assayed in PSY316AUT. Isolation name for each transposon-mutagenized plasmid is indicated.

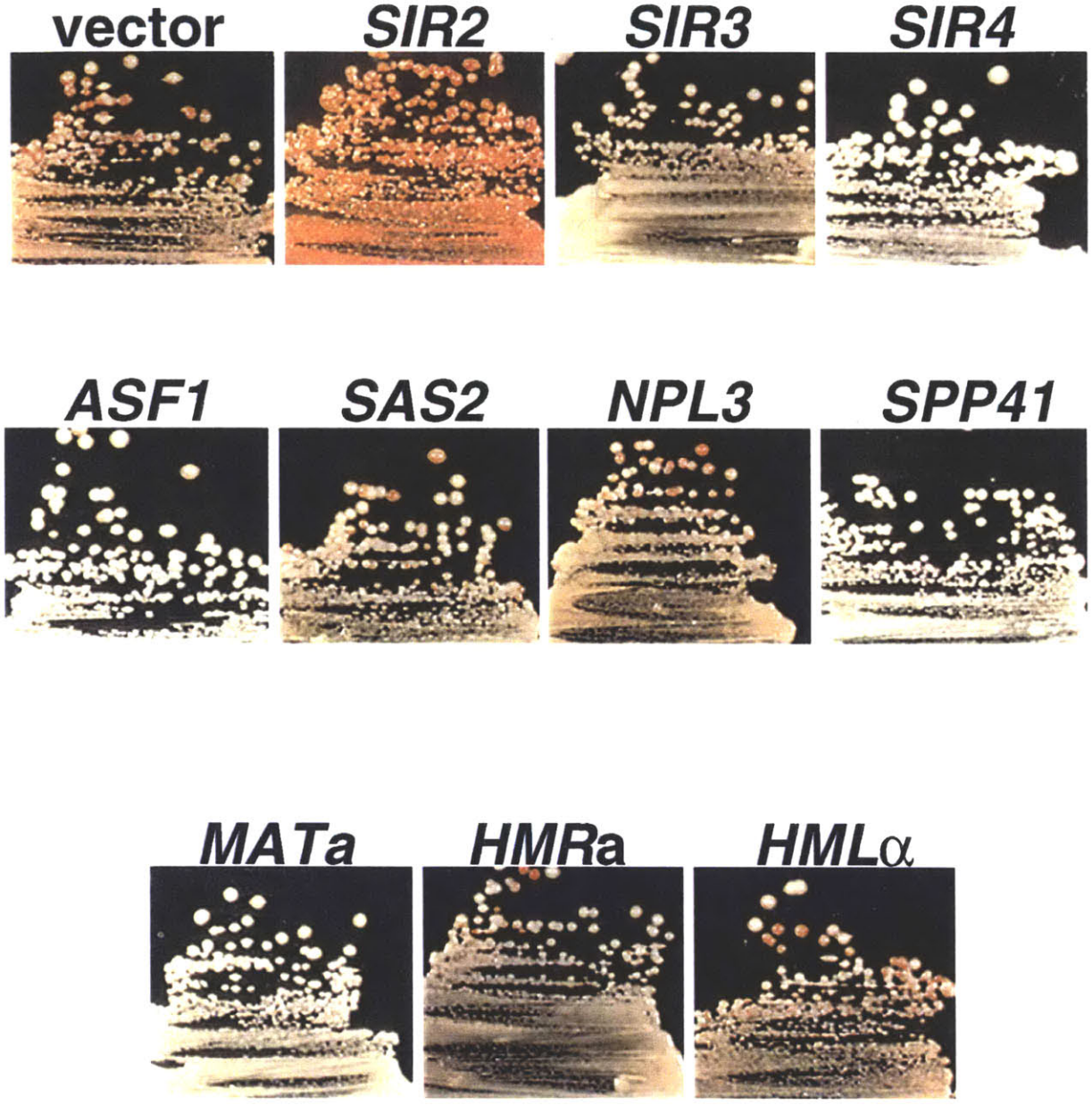

vector
SIR2
SIR3
SIR4
ASF1
SAS2
NPL3
SPP41
MATa
HMRa
HMLα

FIGURE 3- Effect of high-copy telomere desilencing plasmids on telomeric *ADE2* expression. PSY316AUT containing high-copy plasmids bearing the indicated gene was grown on synthetic complete medium lacking leucine and containing low adenine (SC-leu/low ade) to assay the state of telomeric silencing. Red indicates silent *ADE2*. Vector-only control contained the 2μ plasmid pDB20Leu-BglII.

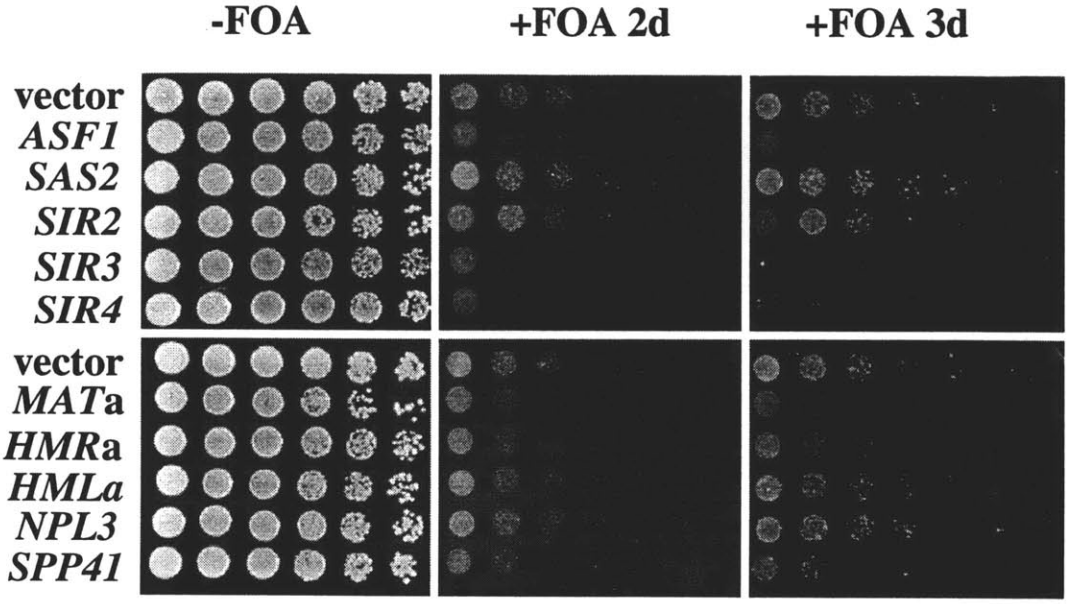
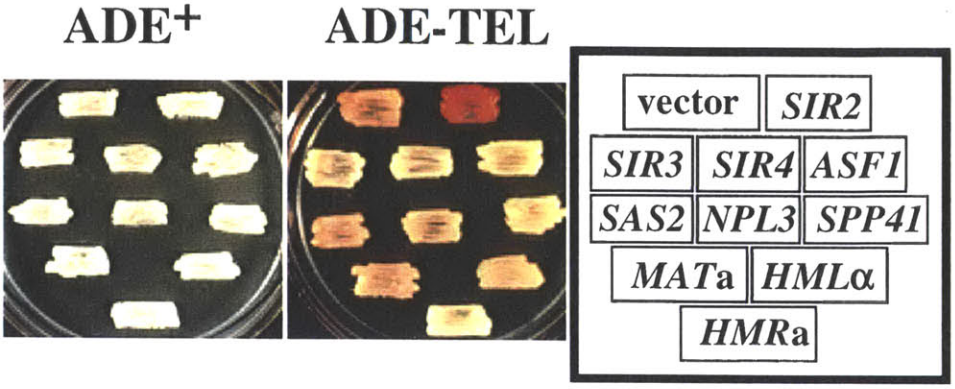
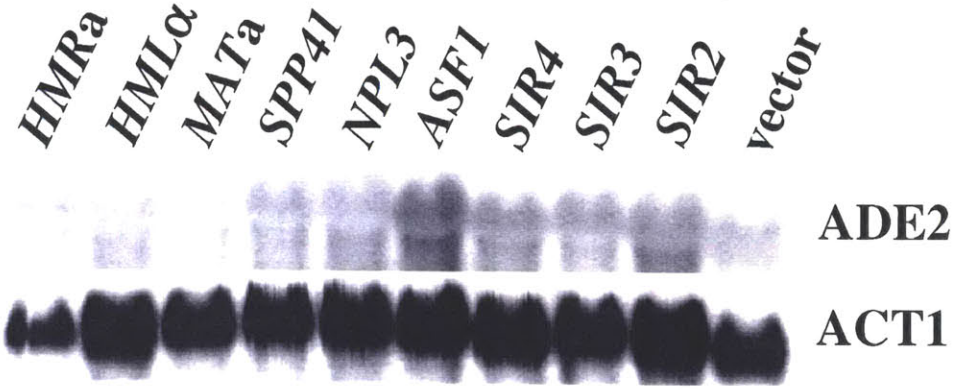

-FOA
+FOA 2d
+FOA 3d
vector
ASF1
SAS2
SIR2
SIR3
SIR4
vector
MATa
HMRa
HMLa
NPL3
SPP41

FIGURE 4- Effect of high-copy telomere desilencing plasmids on expression of telomeric *URA3*. PSY316AUT containing high-copy plasmids bearing the indicated gene was grown in SC-leu medium and 10-fold serial dilutions were spotted onto SC-leu or SC-leu +5 mg/ml 5-FOA to assay expression of telomeric *URA3* marker. Expression of *URA3* results in elevated sensitivity to 5-FOA. SC-leu plates were incubated at 30^{o} for 2 days. SC-leu + 5-FOA plates were grown at 30^{o} for 3 days.

A.


ADE+
ADE-TEL
vector
SIR2
SIR3
SIR4
ASF1
SAS2
NPL3
SPP41
MATa
HMLα
HMRa

B.


HMRa
HMLα
MATa
SPP41
NPL3
ASF1
SIR4
SIR3
SIR2
vector
ADE2
ACT1

C.

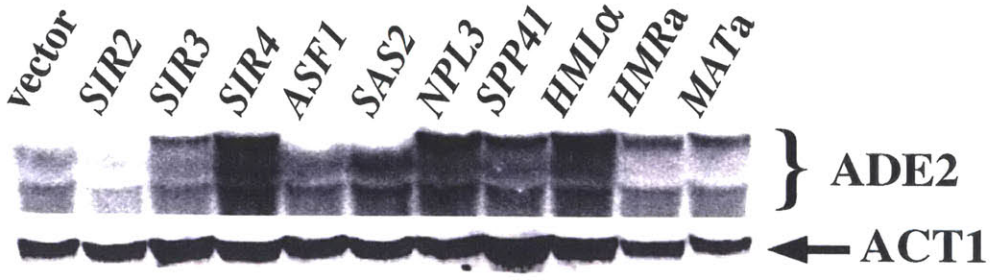
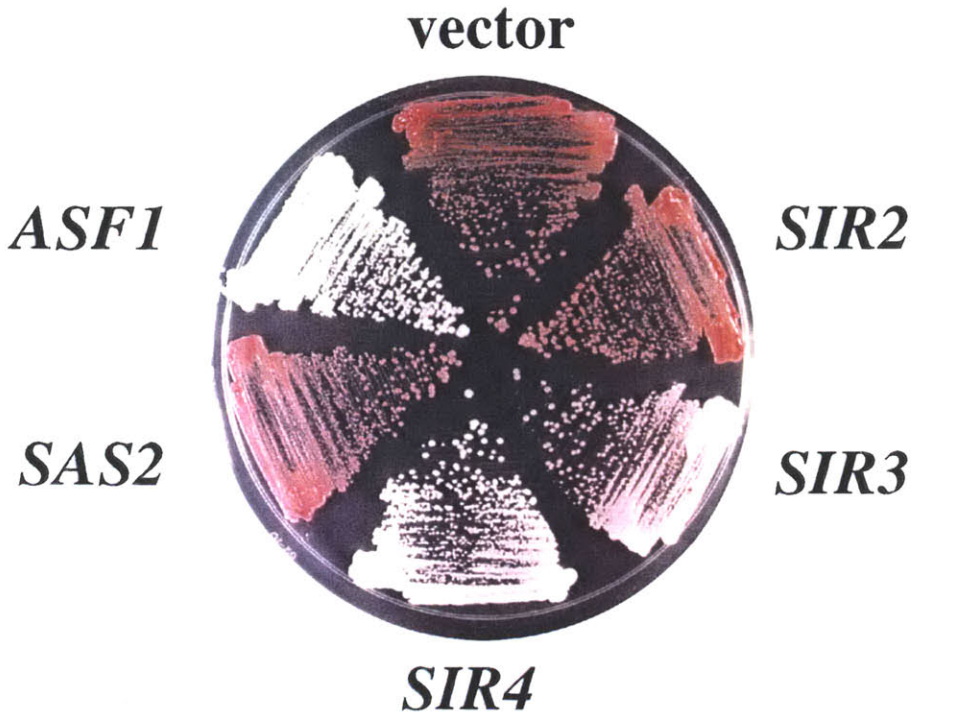

vector
SIR2
SIR3
SIR4
ASF1
SAS2
NPL3
SPP41
HMLα
HMRa
MATa
ADE2
ACT1

FIGURE 5- (A) Effects of high copy desilencers on *ADE2* expression from it native locus. A strain with *ADE2* at its native locus (yCA1), and containing high copy telomere desileencing plasmids, was patched onto SC-leu/low ade medium to assay color formation. PSY316AUT containing desilencing plasmids is included for comparison. (B) Northern blot analysis of *ADE2* expression from its native locus. Total RNA was prepared from yCA1 containing high copy telomere desilencers and probed for *ADE2* mRNA. Equal loading was verified by probing for *ACT1* mRNA. Loading was also confirmed by comparison of ribosomal RNA bands on the ethidium bromide stained agarose gel (not shown). (C) Northern blot analysis of *ADE2* expression from the telomere performed as in (B).


vector
ASF1
SIR2
SAS2
SIR3
SIR4

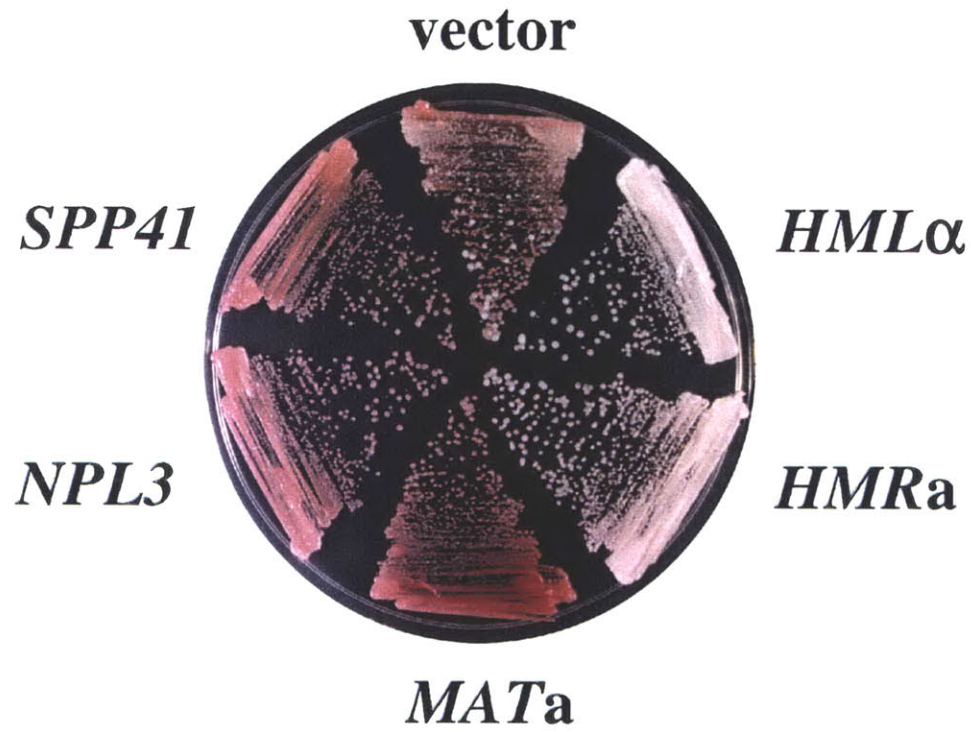

vector
SPP41
HMLα
NPL3
HMRa
MATa

FIGURE 6- Effect of high-copy telomere desilencers on silencing of an *HMR* reporter. Strain ySB1063 contains a *TRP1* gene silenced by wild-type *HMR* silencer and *ADE2* fused to a partial *URA3* promoter silenced by an *HMR* silencer. The *HMR::URADE2* reporter is integrated at the *HIS3* locus. This strain forms red colonies on SC-leu/low ade medium. Strains containing each of the telomere desilencing plasmids were streaked onto SC-leu/lowade medium, grown at 30^{o} for 3 days, and incubated overnight at 4^{o} to assay color formation.

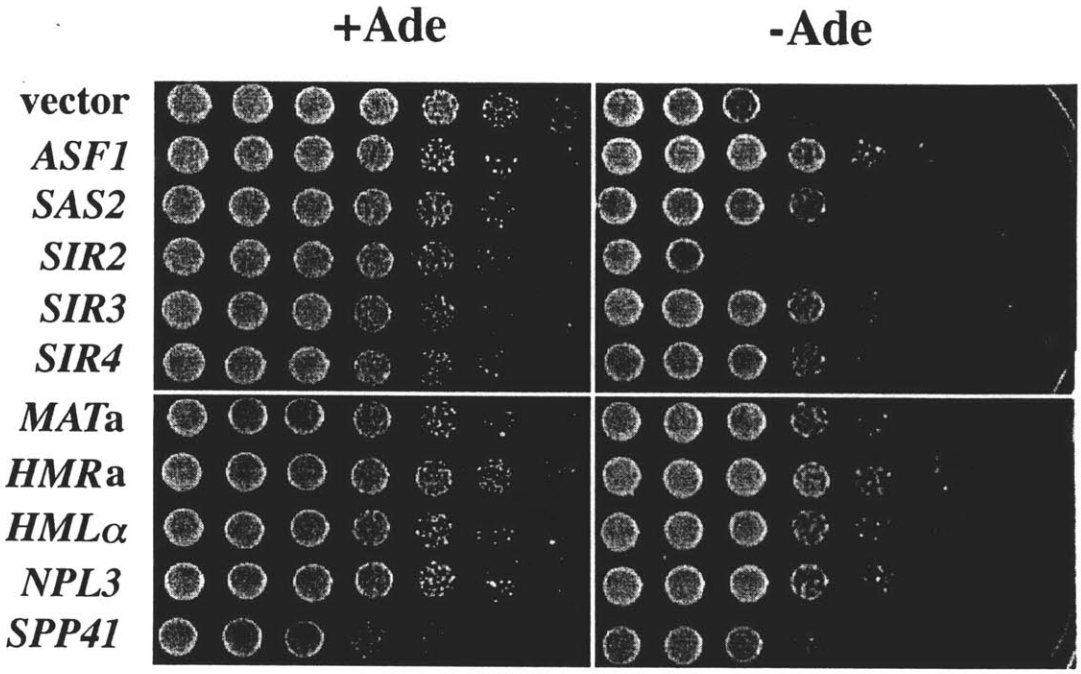
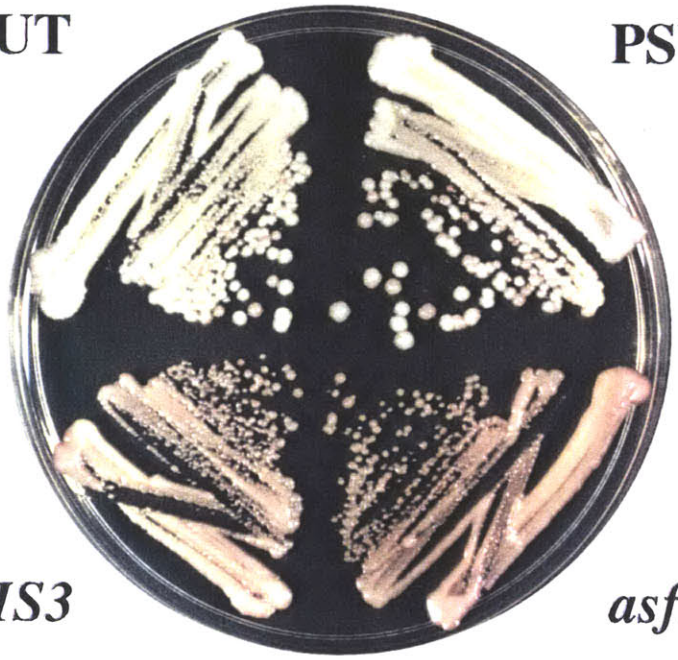

+Ade
-Ade
vector
ASF1
SAS2
SIR2
SIR3
SIR4
MATa
HMRa
HMLα
NPL3
SPP41

FIGURE 7- Effect of high-copy telomere desilencers on silencing at rDNA. W303AR containing high-copy telomere desilencing plasmids was grown in SC-leu and ten-fold serial dilutions were spotted to SC-leu or SC-leu -ade. Silencing of the *ADE2* marker results in a reduced ability to grow on medium lacking adenine.

PSY316AUT

PSY316AUT



asf1::Tn*HIS3*

asf1::Tn*HIS3*

FIGURE 8- Disruption of ASF1 results in enhanced telomere silencing. *ASF1* was disrupted in the telomere silencing reporter strain PSY316AT. Two independent disruption isolates were streaked on YEPD medium and grown at 30^0 for 3 days then incubated at 4^o for two days to allow for development of red color.

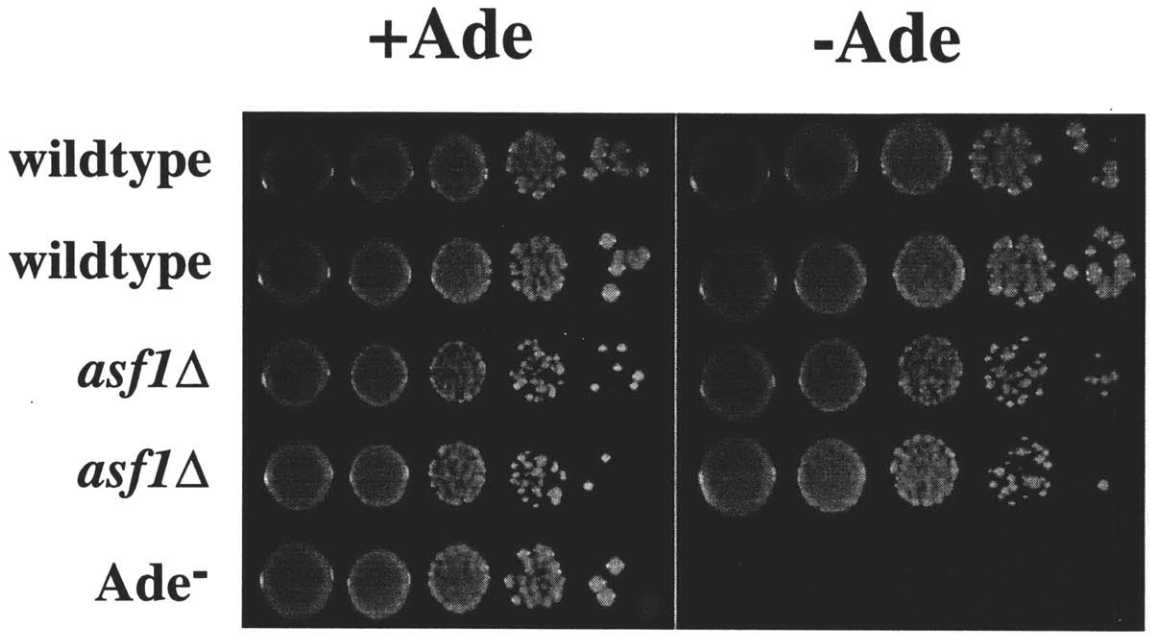

+Ade
-Ade
wildtype
wildtype
asf1Δ
asf1Δ
Ade-

FIGURE 9- Disruption of *ASF1* does not affect silencing at the rDNA. *ASF1* was disrupted in the rDNA silencing reporter strain W303AR, using the Tn*HIS3* mutagenized *ASF1*-containing YEp13 library plasmid as a disruption construct (Materials and Methods). Two independent disruption isolates were used to inoculate overnight YEPD liquid cultures. The overnight cultures were washed, adjusted to OD_{600}= 1 and ten-fold serial dilutions were spotted to synthetic complete medium (SC) or to SC-ade medium to select for *ADE2* expression. Plates were incubated at 30^{o} for 2 days. The Ade^{-} PSY316 was included as a control.

Chapter 3: *MEC1*-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks.

This chapter was previously published:

Mills, K.D., Sinclair, D.A., and Guarente, L. (1999). *MEC1*-dependent redistribution of the Sir3 silencing protein from telomeres to DNA double-strand breaks. Cell. 97: 609-620.

Summary

The yeast Sir2/3/4p complex is found in abundance at telomeres where it participates in the formation of silent heterochromatin and telomere maintenance. Here we show Sir3p is released from telomeres in response to DNA double strand breaks (DSBs), binds to DSBs and mediates their repair, independent of cell mating type. Sir3p relocalization is S phase-specific and, importantly, requires the DNA damage checkpoint genes *MEC1* and *RAD9*. *MEC1* is a homologue of *ATM*, mutations in which cause Ataxia Telangiectasia (A-T), a disease characterized by various neurologic and immunologic abnormalities, a predisposition for cancer, and a cellular defect in repair of DSBs. This novel mode by which pre-formed DNA repair machinery is mobilized by DNA damage sensors may have implications for human diseases resulting from defective DSB repair.

Introduction

Damaged DNA poses a serious risk to the integrity of the cell, while improper repair can be mutagenic or lethal. One catastrophic type of lesion is the DNA double-strand break (DSB), which, if improperly repaired, can directly lead to many types of cancer (Vamvakas et al., 1997). DSBs can be repaired without a loss of information in two ways: homologous recombination and non-homologous end-joining (NHEJ) (Kanaar and Hoeijmakers, 1997). Defects in DSB repair machinery are implicated in a number of inherited human diseases including Nijmengen Break Syndrome (Carney et al., 1998; Varon et al., 1998) and Ataxia Telangiectasia (A-T) (Shiloh and Rotman, 1996; Jeggo et al., 1998).

The gene mutated in A-T, *ATM* (Savitsky et al., 1995), has homology to a family of PI 3-kinase-like genes including human DNA-PK_{CS} (Hartley et al., 1995), *S. pombe RAD3* (Bentley et al., 1996), and *S. cerevisiae MEC1* (Weinert et al., 1994; Kato and Ogawa, 1994) and *TEL1* (Morrow et al., 1995). A-T cells are three to five times more sensitive to agents that generate DSBs (Sullivan and Lyne, 1990; Hittelman and Sen, 1988) and retain a higher residual level of DSBs following irradiation (Zhou et al., 1998; Foray et al., 1997).

ATM has been proposed to play a critical role in the DNA damage response network, by arresting the cell cycle and activating relevant DNA damage repair genes. Evidence suggests that a primary cause of A-T phenotypes is a defect in DSB repair and immunoglobulin class switching (reviewed in Jeggo et al., 1998). Components of mammalian NHEJ machinery have been identified, including the Ku70 and Ku80 end-binding proteins (Mimori et al., 1990; Takiguchi et al. 1996), ligase IV

(Robins et al., 1996; Wei et al., 1995), and DNA-dependent protein kinase, DNA-PK_{CS}, (Hartley et al., 1995). The mechanism by which ATM regulates DNA repair is largely unknown. In addition to DNA repair machinery, cells require a mechanism to prevent cell cycle progression until a lesion is repaired. Cell cycle checkpoints monitor the state of the DNA and arrest cells if damage is detected. In yeast, the DNA damage checkpoint requires at least eight genes: *RAD9*, *RAD17*, *RAD24*, *RAD53*, *MEC1*, *DDC1*, *DUN1* and *MEC3* (reviewed in Weinert, 1998). The products of these genes sense DNA damage, slow the cell cycle, and transduce a signal to repair machinery by mechanisms that are poorly understood. *MEC1*, *RAD9*, and *RAD53* integrate numerous inputs, monitoring chromosomal integrity and DNA replication to coordinate cell cycle progression.

The yeast silent information regulatory complex, composed of Sir2p, Sir3p, and Sir4p, is required for transcriptional repression of the silent mating type loci and genes placed within telomeric regions (Aparicio et al., 1991). Sir proteins are believed to repress transcription by polymerizing across nucleosomes to create an inactive heterochromatic state (Grunstein, 1997). In young cells the Sir complex may be visualized at telomeric foci near the nuclear periphery. However, as cells age, the Sir complex relocalizes to the nucleolus where circular ribosomal DNA molecules accumulate (Kennedy et al., 1997; Sinclair and Guarente, 1997).

Interestingly, the Sir complex has also been implicated in NHEJ. Sir4p physically interacts with Hdf1p (yeast Ku70) and mutation of the Sir complex results in deficient repair of linear plasmids (Tsukamoto et al., 1998; Boulton and Jackson, 1998). In addition, Hdf1p is physically associated with telomeres and is crucial for telomere maintenance and

silencing (Boulton and Jackson, 1998; Gravel et al, 1998; Laroche et al. 1998; Boulton and Jackson, 1996). It was recently indicated that mutations in the *SIR* genes may affect NHEJ indirectly by creating an **a**/α cell type which itself represses NHEJ (Astrom et al., 1999). Here we show that Sir3p acts directly in DSB repair by dissociating from telomeres and associating with broken DNA. This redistribution requires the *MEC1* and *RAD9* checkpoint genes and is cell cycle-dependent. These findings may also have implications for mechanisms of DSB repair in mammals.

RESULTS

Sensitivity of *sir* mutants to DSBs

To investigate the role of the Sir proteins in NHEJ *in vivo*, we utilized a system that produces lesions predominantly addressed by this mode of repair (Lewis et al., 1998). DSBs were introduced by expressing the restriction endonuclease *Eco*RI from a galactose-inducible promoter (Lewis et al., 1998; Barnes and Rio, 1997; Barnes and Rine, 1985) which generated broken DNA ends that are recognized by the Ku heterodimer (Lewis et al., 1998; Barnes and Rio, 1997).

We compared the *Eco*RI sensitivity of *SIR* mutants and homologous recombination (*rad52*) or NHEJ (*hdf1*) mutants. As expected, *hdf1* cells were more sensitive than *rad52* cells to *Eco*RI induction (Figure 1A). Consistent with a role in NHEJ, strains mutant for *SIR2, SIR3* or *SIR4* were at least as sensitive as an *hdf1* strain to *Eco*RI cleavage. As controls, strains containing the plasmid pRS316 exhibited no change in plating efficiency after four hours of growth in medium containing galactose (data not shown).

A plasmid-based transformation repair assay provides another sensitive test of NHEJ efficiency. Under selective conditions, cells transformed with linearized centromeric plasmids will not form colonies unless the plasmid is recircularized. The efficiency of recircularization is expressed as a fraction of the transformation efficiency of linear to circular plasmids. Similar to survival following induction of chromosomal breaks, NHEJ repair of linear plasmids requires *SIR* genes (Tsukamoto et al., 1997; Boulton and Jackson, 1998). Because cell mating type has been implicated in the regulation of DNA repair (Heude and Fabre, 1993; Astrom et al, 1999), we wished to reassess the influence of cell type on NHEJ. The efficiency of plasmid recircularization was compared in isogenic wild type, *hdf1*, *sir,* and *sir hmlα* strains. Deletion of *HMLα* in the *sir2*, *sir3*, or *sir4* strains restores **a**-mating type proficiency. Consistent with published results (Tsukamoto, et al. 1998; Boulton and Jackson, 1998), strains mutant for *hdf1*, *sir2*, *sir3*, or *sir4* transformed 20- to 50-fold less efficiently with linear plasmid than wild type (Figure 1B). Transformation efficiencies of the *sir hmlα* double mutants were 2- to 5-fold greater than those of the *sir* single mutants, but still substantially less than that of wild type. This indicates that cell mating type has only a partial influence on the repair of linear plasmid DNA.

The contribution of mating type to DSB sensitivity was determined by comparing survival of *Eco*RI induction in wild type, *hdf1*, *sir2*, and *sir2 hmlα* strains (Figure 1C). The *sir2* and *hdf1* strains were 5-10 fold more sensitive than wild-type after as much as 20 hours of *Eco*RI induction. Deletion of *HMLα* from the *sir2* strain did not confer any greater resistance to *Eco*RI expression. Importantly, these results demonstrate that the sensitivity of *sir* mutants to chromosomal DSBs is not

due to cell mating type effects and suggests a direct role for *SIR2*, *SIR3*, and *SIR4* in facilitating DSB repair.

To verify that the lethality in *SIR* mutant strains was associated with DNA cleavage, genomic DNA from wild type and mutant cells was analyzed following expression of *Eco*RI (Figure 1D). By five hours of induction, genomic DNA exhibited cleavage, reducing the average size of DNA fragments from 45 kb to 33 kb. By 10 hours the average fragment size in *sir2*, *rad52* and *hdf1* mutant strains was approximately 20 kb. The average size of the genomic DNA from the *sir2 rad52* strain after *Eco*RI induction was approximately 10 kb smaller than from the single mutants, indicating that this strain was particularly deficient in DSB repair.

Cell cycle dependent relocalization of Sir3p

To test the possibility that the Sir complex can relocalize in response to DNA damage, we induced DSBs by expressing *Eco*RI, and determined the resulting distribution of Sir3p. After three hours of *Eco*RI induction, a diffuse but intense pattern of Sir3p staining was observed throughout the nucleus in approximately 20% of the cells in an asynchronous culture (data not shown). In contrast, greater than 99% of cells retained telomeric foci in the uninduced control. To determine if those cells showing redistribution of Sir3p were in a particular phase of the cell cycle, we arrested cells in the G1 phase with α-factor and transiently induced *Eco*RI by changing the carbon source to galactose (Figure 2). Cells lacking inducible *Eco*RI were used as a control (Figure 2A). Sir3p localization was then monitored by immunofluorescence after cells were released from the α-factor block, shifted to glucose media, and allowed to progress through the ensuing cell cycle. Cells retained a telomeric staining pattern

as long as they were held in G1, even under continuous *Eco*RI induction (not shown). When damaged cells were allowed to pass from G1 to S phase, Sir3p redistributed in a diffuse pattern throughout the nucleus in greater than 90% of the cells (Figure 2B). As these cells exited S phase, Sir3p staining returned to its telomeric pattern.

The above results show that Sir3p is released from telomeres in response to *Eco*RI generated DSBs. Further, the release only occurs upon entry into S phase, after which normal telomere-localization returns in G2.

A single DSB can elicit Sir3p redistribution

As a further test that Sir3p responds specifically to DSBs, a single break was introduced into chromosome III at the *MAT* locus using a galactose inducible HO endonuclease. To prevent DSB repair by homologous recombination or gene conversion using homologous sequences at *HML* and *HMR*, we utilized a strain deleted for these loci (gift of S.E Lee and J. Haber). Unlike the repair of the *Eco*RI breaks, the repair of the chromosome III DSB in this strain is inefficient, so damaged cells traverse S phase and arrest in G2 (Lee et al., 1998).

As with *Eco*RI, induction of a single DSB in G1 resulted in redistribution of Sir3p following passage into S phase (Figure 3A). Restoration of a telomeric pattern after S phase was less evident after HO damage than after *Eco*RI damage, perhaps owing to the persistence of DNA break into the G2 phase. These results provide further evidence that Sir3p redistributes in response to DSBs incurred during G1. Since a single DNA break is sufficient to cause the dispersal of the entire cellular complement of Sir3p, the response cannot simply be due to titration of Sir

proteins to DNA breaks by affinity. This result implies that the release of telomere-bound Sir proteins is an active process that may require sensitive DSB sensors.

DSB specificity of Sir3p redistribution

To further characterize the types of DNA lesion that elicit the Sir3p response, we performed identical cell cycle analyses using a variety of DNA damaging agents (Figure 3, A-C). The radiomimetic agent, methylmethane sulfonate (MMS) produces 3-methyl adenine adducts at a high frequency but is also known to produce other types of lesions, including DSBs (Schwartz, 1989; Mitchel and Morrison, 1987). The radiomimetic anti-tumor agent, bleomycin, generates DSBs as its major product (Hecht, 1986). Identical to the effect seen with *Eco*RI damage, treatment with 0.02% MMS (Figure 3A) or 20 μg/ml bleomycin (Figure 3C) in G1 resulted in redistribution of Sir3p in S phase.

To determine whether the redistribution of Sir3p affects the state of telomeric silencing we examined the expression of a telomeric *ADE2* gene in the presence of the DSB-inducing agent bleomycin. Yeast cells that do not express *ADE2* form red-pigmented colonies on medium containing low levels of adenine. Cells that contain *ADE2* in proximity to a telomere develop red-sectored colonies due to heritable Sir-dependent transcriptional silencing (Gottschling et al., 1990; Aparicio et al., 1991). In support of immunofluorescence data that shows that Sir3p is released from telomeres following induction of DSBs, transcriptional repression of the telomeric *ADE2* gene was compromised when cells were grown in the presence of bleomycin (Figure 3D). Since UV damage is predominantly repaired by nucleotide excision, UV irradiation was not expected to evoke

Sir3p redistribution. Consistent with this prediction, cells irradiated with UV retained a telomeric pattern of Sir3 staining during arrest and passage through S phase (Figure 4A). We also examined Sir3p relocalization following γ–irradiation. As with UV irradiation, Sir3p did not redistribute during any phase of the cell cycle following γ–irradiation during G1 (Figure 4B). Interestingly, only those agents to which Ku-deficient cells are sensitive (Moore and Haber, 1996; Siede et al., 1996; Teo and Jackson, 1996; Mages et al., 1996; Milne et al., 1996) cause the redistribution of Sir3p.

Sir3p redistribution requires the DNA damage checkpoint

Mutation of genes involved in the yeast DNA damage checkpoint results in defects in both cell cycle arrest and transcriptional induction of repair genes. To examine whether the DNA damage checkpoint pathway is required for release of the Sir complex, we followed Sir3p localization in *mec1-1* (Figure 5A) and *rad9Δ* (Figure 5B) strains after induction of DSBs. Under the conditions used here, *mec1-1* or *rad9Δ* strains proceeded through the cycle only slightly faster than wild type (data not shown). Cells in similar stages of S phase progression are compared in Figure 5. In both mutant strains, Sir3p remained telomeric throughout the cell cycle. In isogenic wild type strains, *Eco*RI induction resulted in normal Sir3p redistribution (not shown). Thus, in addition to their roles in cell cycle arrest and transcriptional activation, Mec1p and Rad9p communicate with telomere bound proteins, resulting in redistribution of the Sir complex. We also examined whether another PI 3-kinase-related protein, *TEL1* (Morrow et al., 1995), is involved in Sir3 redistribution.

In a *tel1Δ* strain, the redistribution of Sir3p following *Eco*RI induction was similar to the wild type (data not shown).

Sir3p dissociates from telomeric DNA

To obtain independent confirmation of Sir3p dissociation from telomeric DNA following induction of DSBs, we utilized chromatin immunoprecipitation (ChIP) analysis. Sir3p has previously been shown to physically associate with telomeric DNA up to at least 5 kb from chromosome ends (Hecht et al., 1996; Strahl-Bolsinger et al., 1997). Sir3p-associated chromatin was crosslinked in vivo, extensively sheared, immunoprecipitated, and analyzed by PCR. Telomeric DNA was analyzed using primer pairs to detect target sequences 300, 3500, or 5800 bp from the right end of chromosome VI (TEL-300, TEL-3500, TEL-5800, respectively; Figure 6A). To determine the specificity of the antibody for Sir3p associated chromatin, wild type or *sir3Δ* strains were subjected to ChIP analysis with the TEL-300 primer pair. The absence of detectable product after PCR from the *sir3Δ* precipitate demonstrates that the presence of Sir3 protein is required to immunoprecipitate telomeric DNA with the anti-Sir3p antibody (Figure 6B).

The association of Sir3p with telomeric chromatin was monitored both before and after induction of *Eco*RI expression and strain mutant for *RAD9* was included as a control. Following DSB induction, the ability to immunoprecipitate Sir3p-associated telomeric heterochromatin was reduced substantially in wild type but to a much lesser degree in the *rad9Δ* mutant using the TEL-5800 primers (Figure 6B). This finding corroborates the immunofluorescence data and indicates that Sir3p leaves telomeres in response to DSBs. Dissociation of Sir3p from telomeric

heterochromatin was more easily detected further from the chromosome end, with maximal difference between the wild type and *rad9Δ* observed using the TEL-5800 primers, and a minimal difference using the TEL-300 promers. This may indicate that silent heterochromatin disassembly begins at telomere-distal regions and proceeds toward the end of the chromosome.

Association of Sir3p with a DSB

Because genetic experiments have provided strong evidence that the Sir protein complex is directly involved in NHEJ, we wished to determine if Sir3p can physically associate with a DSB. Primer pair EcoP-1 ('proximal') detects a target sequence between *Eco*RI sites within a cluster on chromosome XV. To control for the specificity of Sir3p association with DSBs, immunoprecipitates were also probed with three primer pairs distal to (i.e. far from) *Eco*RI sites. The EcoD-1 and EcoD-2 ('distal') primer pairs detect chromosome XV target sequences approximately 15 kb from a cluster of five *Eco*RI sites and the EcoD-3 primer pair detects a chromosome III target sequence 3.1 kb from the nearest *Eco*RI site. In these experiments, an equal amount of a plasmid PCR template was added to each reaction as an internal control for PCR efficiency and loading.

To determine if Sir3p could associate with DNA adjacent to a DSB, cells were arrested in G1, damaged by *Eco*RI induction, and released into S phase. Samples for ChIP analysis were taken 45 minutes after release into S phase, at a time when maximal Sir3p release was observed. As controls, additional samples were prepared from an asynchronous, untreated culture and from G1 arrested cells in the experiment described above. Probing immunoprecipitates with EcoD-1 and EcoD-2

demonstrated that Sir3p was not associated with DNA in these regions in either a wild type or a *rad9Δ* strain either before or after induction of *Eco*RI (Figure 6C). As in Figure 6C, target sequences distal to an *Eco*RI site were not detected in immunoprecipitates with the EcoD-3 primer pair, even after release into S phase (Figure 6D, upper panel). Strikingly, the EcoP-1 target sequence was detected in immunoprecipitates after release into S phase, but not from untreated or G1-arrested cells (Figure 6D, lower panel). Consistent with the finding that mutation of *RAD9* abolishes Sir3p redistribution, deletion of *RAD9* abolished the association of Sir3p with DNA near the *Eco*RI site cluster (Figure D, *rad9*).

DISCUSSION

One central component of heterochromatin in yeast is Sir3p, a protein that interacts with histones, other silencing factors, and is a target for a variety of cellular signals (reviewed in Stone and Pillus, 1998). The finding that Sir proteins are sequestered at telomeres led to the model that telomeres are sinks for Sir proteins that regulate silencing at other loci (Smith et al. 1998; Marcand et al. 1996). The results described here show that Sir3p responds specifically to DSBs. We propose that telomeres are reservoirs for DNA repair proteins that can be mobilized to other sites in the genome in response to DNA damage, where they may perform structural functions similar to those at telomeres.

One possible explanation for the diffuse Sir3p staining pattern is that DSBs induced changes in Sir3p conformation, revealing a previously masked pool of the protein. However, two observations argue against this possibility. First, we used polyclonal antibody directed against full-length

Sir3p minimizing the chance that detection will be substantially altered due to conformational changes. Second, identical results have been obtained using Sir3p tagged with green fluorescent protein, without the use of immunostaining (D.A.S, K.D.M, H. Fraser, D. Moazed, L.G., unpublished results).

Although the native yeast endonuclease HO has been used in the study of DSB repair, we chose to utilize *Eco*RI to analyze NHEJ in vivo for two reasons. First, like HO, *Eco*RI generates DNA ends that can be efficiently recognized by NHEJ machinery. Importantly, this machinery is also required for resistance to *Eco*RI expression, in vivo. Second, unlike HO, there is no evidence for a DNA repair mechanism dedicated specifically to the repair of *Eco*RI-generated DSBs. Cleavage by HO at the *MAT* locus is the first step in the specialized process of mating type switching by gene conversion. Following cleavage, the HO endonuclease may not rapidly release from a DNA end, perhaps forming higher-order stuctures at the break site (Jin et al., 1997). Additionally, it is possible that DNA repair mechanisms unique to mating type interconversion may interfere with processing by the NHEJ machinery.

Direct role for Sir proteins in repair of DSBs

Previous work has demonstrated that *SIR2*, *SIR3*, and *SIR4* are required for the efficient recircularization of linear plasmids by NHEJ (Tsukamoto et al. 1998; Boulton and Jackson, 1998). More recently it was suggested that the role of *SIR* genes in NHEJ was mediated indirectly by a change in the cell mating type in *sir* mutants (Astrom et al., 1999). Here we provide three lines of evidence for a direct role of the Sir proteins in DSB repair. First, the simultaneous expression of **a** and α mating type

information in wild type haploid cells only partially influences the efficiency of repair of linear plasmids. Also, in haploid cells lacking cryptic mating type loci, deletion of *SIR* genes reduces the efficiency of NHEJ about three-fold. Second, deletion of *SIR2* renders cells hypersensitive to the induction of *Eco*RI, and this hypersensitivity is not due to the cell mating type. Third, Sir3p can be crosslinked to DNA near sites of *Eco*RI cleavage, but not to distal DNA, implying that a redistributed Sir complex physically associates with DNA breaks.

It is possible that the difference between our findings and those of Astrom et al. (1999) reflect the contribution of *RAD5*, a gene known to affect the type of repair at a DSB (Ahne et al., 1997). W303-1A, which was used in their studies, contains a point mutation in *RAD5* (*rad5-535*) that renders the protein partially inactive (Fan et al., 1996). We have found that expression of **a** and α mating type information in *rad5-535* can influence the outcome of repair events involving linear plasmid DNA (K. M. and L. G., unpublished results).

Reorganization of Sir3p heterochromatin following DNA damage

Immunofluorescence showed that Sir3p redistributes during S phase following induction of DSBs in G1, suggesting that the Sir protein complex is released from telomeres to diffuse throughout the nucleus. Chromatin immunoprecipitation from wild type cells confirmed that Sir3p is released from telomeric DNA substantially at five kb from the end of chromosome VI following DNA damage, but less substantially 300 base pairs from the end of the chromosome.

Cells defective in *hdf1* and thus NHEJ are sensitive to treatment with *Eco*RI, HO endonuclease, MMS, and bleomycin but not UV- or γ-irradiation. Only those DNA damaging agents to which *hdf1* strains are sensitive provoke Sir3p redistribution, showing a clear correlation between the efficacy of NHEJ in repairing DNA damage and the redistribution of the Sir complex. In addition, the recent results of Martin et al. (co-submitted) indicate that Ku is also released from yeast telomeres in response to DSBs. A picture emerges that telomeres are storage sites (Marcand et al., 1996; Maillet et al., 1996) for pre-formed repair complexes involved in NHEJ. Such a mechanism of mobilization could respond more readily than those that require transcriptional induction and protein synthesis.

A direct role of Sir proteins in NHEJ may reflect a function of heterochromatin in isolating broken DNA from the replication machinery or suppressing deleterious recombinational events. Alternatively, Sir proteins may act as a splint, binding to nucleosomes surrounding the break to provide chromosome stability or a more constrained substrate for DNA ligase VI.

MEC1 and *RAD9* control Sir3p release from telomeres

The release of Sir3p in response to DSBs requires the Mec1p pathway since mutations in *MEC1* or *RAD9* prevent release. Mec1p is known to phosphorylate Rad9p in response toDNA damage in all phases of the cell cycle. Although many forms of DNA damage can stimulate the Mec1p pathway (Emili, 1998), Sir3p release appears to be specific to DSBs. In order to address this specificity we propose the model in Figure 7. First, cells sense DSBs via the Mec1p checkpoint signal transduction

pathway. Second, a unique signal originating from DSBs provokes the release of the Sir complex from telomeres. Third, DSBs nucleate the binding of a possibly altered form of the Sir/Ku complex. Release of the complex and subsequent binding to DSBs occurs selectively during S phase, a point we consider further below. ChIP assays suggest that Sir proteins peel off the chromosome ends starting from sites most distal to telomeres. As further evidence for an active signaling mechanism, a single DSB generated by the HO endonuclease can result in Sir release. Since the majority of Sir3p is released from telomeres following the induction of a single DSB, we presume that only a fraction of the diffuse Sir3p pool is recruited to each DNA break.

Because Mec1p-pathway stimulation is neither cell cycle nor DSB specific we propose an additional signal during S phase that recognizes DSBs and elicits the release of the Sir complex from telomeres. This cell cycle specificity may help explain the findings of Moore and Haber (1996) who noted that the repair efficiency of a chromosomal DSB was 30 times higher when HO was expressed throughout the cell cycle than when HO was expressed in G1.

Implications for DNA repair in mammals

Mutations in the *ATM* gene cause A-T, a disease characterized by various immunological and neurological abnormalities, an increased incidence of cancer, and a dramatic over-reaction to DSB-inducing agents. At the cellular level, A-T fibroblasts are also specifically sensitive to DSB-inducing agents, but not UV (Taylor et al., 1994; Taylor et al., 1975). This sensitivity appears to be due, in part, to an inability to efficiently repair DSB, which persist longer in these cells following damage (Foray,

et al., 1997; Pandita et al., 1992; Blocher et al., 1991). Our findings suggest that another defect in A-T cells may be the inability to mobilize the NHEJ machinery in response to DSBs.

In summary, our findings show that the Sir protein complex is directly involved in NHEJ and illuminate a pathway by which pre-existing repair proteins are rapidly mobilized from telomeres to DSBs. This pathway links checkpoint functiona and heterochromatin factors directly to DNA repair and may have implications for the understanding of human diseases arising from defective DSB repair.

MATERIALS AND METHODS

Yeast strains and plasmids

Yeast strains are listed in Table 1A. W303AR was constructed by integrating plasmid pDS40 into the rDNA of W303-1A. The *rad5-535* allele of W303AR (Sinclair and Guarente, 1997) was corrected by integrating plasmid pBJ6 which contains the *RAD5* gene on a 4.3 kb *Hin*dIII/*Bam*HI fragment in vector Ycplac33 (*URA3*) to generate W303AR-*URA3*. The resulting strain was selected for loss of *URA3* by passage over 5-fluoroorotic acid to generate W303AR5. *SIR2* was disrupted by integration of plasmid pC369, containing the *TRP1* gene inserted into the *Bgl*II site of a *SIR2 Hin*dIII fragment in YIp5. *SIR3* was disrupted by integration of plasmid pSIR3.4, containing the *HIS3* gene inserted into the *Bgl*II site of a 1.8 kb *Bam*HI fragment of *SIR3* in pBluescript. *SIR4* was disrupted by integration of pMM10.7-*HIS4* digested with *Sph*I/*Cla*I (Marshall et al., 1987). *RAD52* was disrupted by integration of pSM20, containing the *LEU2* gene inserted into the *Bgl*II

site of a 2 kb *RAD52* fragment. *RAD9* was disrupted by integration of a PCR product containing the kanamycin resistance gene (Lee et al., 1998). DSY1070 (*tel1::URA3*) was a segregant of a cross between W303-1A and W1907-4A (Zhao et al., 1998). The galactose-inducible *Eco*RI plasmid, YCpGAL:RIb (Barnes and Rine, 1985), was maintained in cells by selection in synthetic complete yeast medium lacking uridine (SC-URA) and containing 2% glucose (SC-URA+glu). *Eco*RI expression was induced by transfer of cells from SC-URA+glu to SC-URA medium containing 3% galactose as the sole carbon source (SC-URA+gal). Induction was typically carried out for 4-5 h at 30°C.

Survival assays

To test survival following induction of DSBs, all strains were treated identically. Cells containing either YCpGAL:RIb or the control plasmid pRS316 were pre-grown at 30°C overnight in SC-URA+glu. Overnight cultures were diluted 1:50 into SC-URA+glu medium and grown approximately 3 h at 30°C. Cultures were then split and centrifuged to pellet cells. Pellets were washed with sterile water twice then resuspended in either SC-URA+glu or SC-URA+gal and cultured at 30°C. Transfer into SC-URA gal medium defined the zero time-point. Aliquots were taken at the indicated times after induction, cell titer was determined by counting using a hemacytometer, and cells were plated to rich medium containing 2% glucose (YPD). Survival was determined as CFU divided by cell titer at each time-point.

Plasmid end-joining assays

Plasmid end joining assays were performed using the yeast shuttle vectors pRS316 or pRS424. Plasmids were linearized within the polylinker using *Sac*I to provide DNA ends that lacked extensive homology with the yeast genome. Yeast cells were transformed using 150 ng of circular plasmid or 300-450 ng of linearized plasmid by standard lithium acetate transformation procedure with 50 μg carrier DNA (salmon sperm DNA). Cells were plated onto SC-URA medium to select for pRS316, or SC medium lacking tryptophan (SC-TRP) to select for pRS424. Transformation efficiency is expressed as CFU with linear plasmid divided by CFU with circular plasmid. Transformation efficiency in all mutant strains is normalized to the transformation efficiency in the wild type strain.

Immunoflourescence and FACS

Polyclonal antibodies directed against Sir3p were generated in rabbits (Covance). Full length Sir3p was expressed in insect cells using a baculovirus expression system (R. Lipford and S. Bell) and used for immunization. Antibody was affinity purified using the immunizing antigen. Cells were prepared for immunofluorescence by fixation in 3.7% formaldehyde. Fixed cells were washed once with 1 ml cold YPD containing 1M sorbitol (YPD-sorb) and resuspended in 1 ml cold YPD-sorb. Spheroplasts of fixed cells were obtained by treatment with Zymolyase for 30 minutes at 30^{o}C. Fixed spheroplasts were analysed by immunofluorescence essentially as described (Gotta et al., 1996). Digital images were obtained using a Hammamatsu CCD camera controlled by OpenLab image acquisition software.

Preparation of cells for fluorescence-activated cell sorting (FACS) was performed as described in Bell et al. (1993) with the following modifications. Between 10^6 and 10^7 cells were harvested, resuspended by the dropwise addition of 70% EtOH, and incubated at 4°C for 6-48 h. Cells were pelleted by centrifugation for 1 min in a microcentrifuge (12 000xg) and resuspended in 1 ml of 50 mM NaCitrate buffer (pH 7.4). Cells were dispersed by sonication and resuspended in 1 ml 50 mM NaCitrate buffer (pH 7.4) containing 250 µg/ml RNase A, and incubated for 2 h at 55°C. Proteinase K (50 µl of 20 mg/ml) was added and cells were incubated for another 2 h at 55°C. Propidium iodide (1 ml of 16µg/ml) was added and FACS was performed on a Beckman-Dickson FACScan using CellQuest software.

Chromatin Immunoprecipitation

Yeast were grown in 100ml SC-URA+glu to OD_{600}=1.2. For cell cycle analyses, cells were arrested in G1 by treatment with 26µg/ml α-mating pheromone (Sigma Co,). *Eco*RI expression was induced by transfer to SC-URA+gal containing 26µg/ml α-mating pheromone for 4 hs, followed by release into fresh SC-URA+glu. Immunoprecipitation of crosslinked DNA was performed essentially as described (Strahl-Bolsinger et al. 1997) using polyclonal rabbit anti-Sir3p antibodies. PCR analysis of immunoprecipitated DNA was performed in 100 µl reaction volume using 100 pmol primer. DNA from pre-cleared whole-cell extract (designated "input") was used at a 1:10,000 dilution and immunoprecipitated DNA was used at a 1:20 or a 1:100 dilution. PCR reaction parameters were as described (Strahl-Bolsinger et al. 1997) and oligonucleotides are listed in Table 1B. PCR products were analyzed by electrophoresis on a 2%

agarose gel and visualized by ethidium bromide staining. ChIP analysis of telomeric DNA was performed as described above, except cultures were not cell cycle arrested prior to induction of *Eco*RI expression, which was carried out for 4-6 h.

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TABLE 1A
Yeast strains

Strain	Genotype
W303-1A	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 rad5-535*
JKM139	*MAT***a** *ade1 leu2-3,112 lys5 trp1::hisG ura3-52 hml::ADE1 hmr::ADE1 ade3::GAL-HO*
YMP848	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 rad5-535 mec1-1*
DLY259	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15 rad5-535 mec2-1*
PSY316	*MATα ura3-53 leu2-3,112 his3-Δ200 ade2-101 can1-100*
PSY316AT	*MATα ura3-53 leu2-3,112 his3-Δ200 ade2-101 can1-100 ADE2*-TEL V-R
W303AR5	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5*
MMY37	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 hdf1::HIS3*
DSY961	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 rad52::URA3*
DSY962	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir2::TRP1 rad52::URA3*
DSY1034	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir2::TRP1*
KMY285	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir2::TRP1 hml::LEU2*
DSY924	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir3::HIS3*
DSY880	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir3::URA3*
KMY286	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir3::URA3 hml::LEU2*
DSY1036	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir4::HIS3*
KMY290	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 sir4::HIS3 hml::LEU2*
KMY284	*MAT***a** *ade2-1 leu2-3,112, can1-100, trp1-1 ura3-52, his3-11,15,* rDNA-*ADE2, RAD5 rad9::kanR*

TABLE 1B
Oligonucleotide sequences.

Oligonucleotide	Sequence
TEL-300.fwd	GGATATGTCAAAATTGGATACGCTTATG
TEL-300.rev	CTATAGTTGATTATAGATCCTCAATGATC
TEL-3500.fwd	TGATTCTGCTTTATCTACTTGCGTTTC
TEL-3500.rev	AGAGTAACCATAGCTATTTACAATAGG
TEL-5800.fwd	TGAAAGTGCCAATTTGCTCATCAGTGC
TEL-5800.rev	AAAGCCACTTTGTTCATCTGGATTACG
EcoD-1.fwd	GTAGTTCGTTAGGTATGGACATTGATTTGGCC
EcoD-1.rev	AAATGAAATGTATTGGGGCCTAGGTTCGCA
EcoD-2.fwd	TGTCTCTGTTGTGGTAGCTTTGATCGCATG
EcoD-2.rev	CCCTCCAATTCGTTAAGTCTATTGCAGTAATTCC
EcoD-3.fwd	TCTACTTGCCTCTTTTGTTTATGTC
EcoD-3.rev	GATCCAACAACAACCTAGAGTAATG
EcoP-1.fwd	GAATATTAGTATCGTGAGAGATATCGG
EcoP-1.rev	GGGTATGCCATTTTCATCCCTAAGTAC
HIS3.fwd	AAAAAGGCCTGGAAGTCATAACACAGTCCTT
HIS3.rev	AAAAACTTAAGAATAATCGGTGTCACTACATA

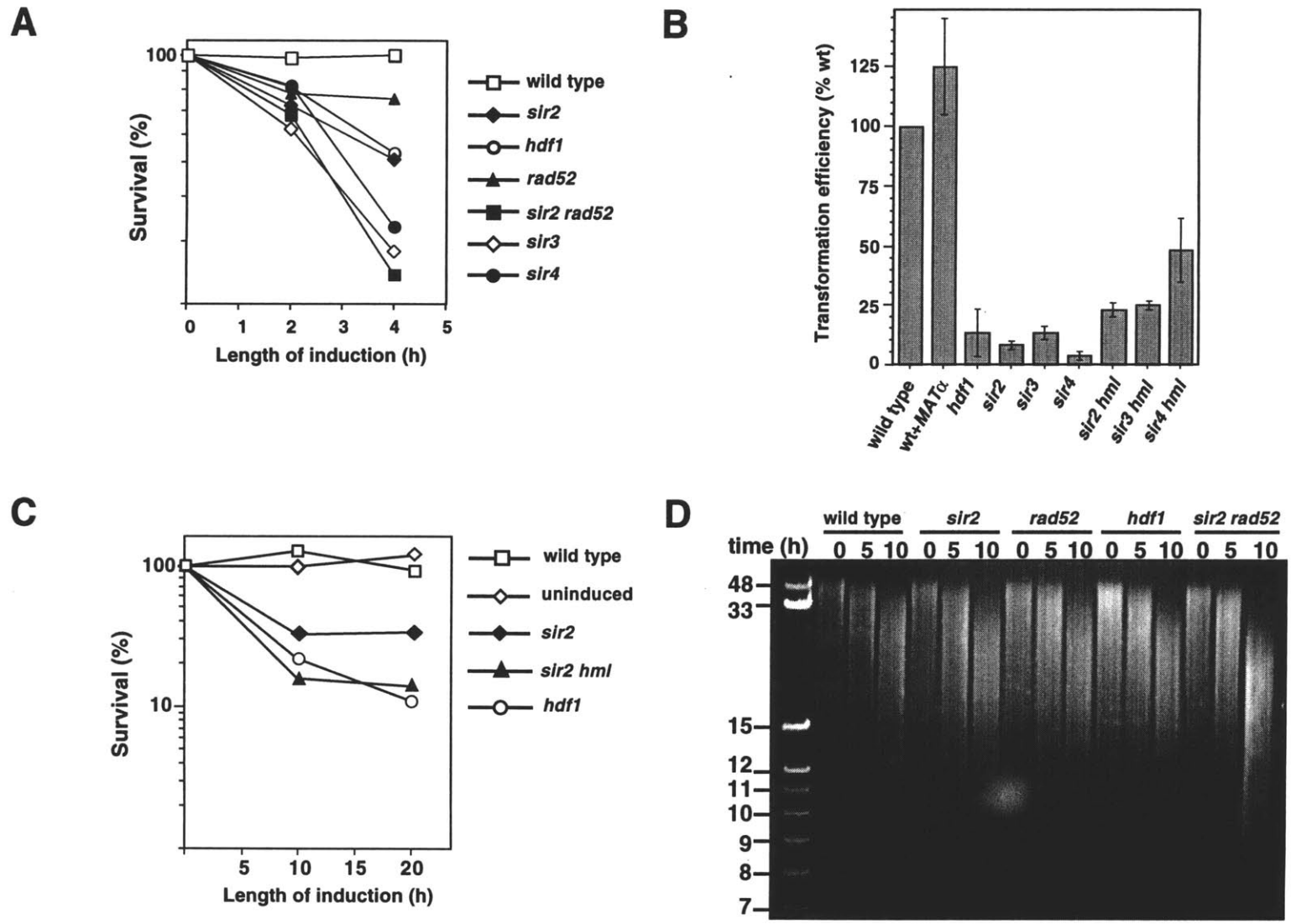
A
100
Survival (%)
0 1 2 3 4 5
Length of induction (h)
wild type
sir2
hdf1
rad52
sir2 rad52
sir3
sir4
B
Transformation efficiency (% wt)
125
100
75
50
25
0
wild type
wt+MATα
hdf1
sir2
sir3
sir4
sir2 hml
sir3 hml
sir4 hml
C
100
10
Survival (%)
5 10 15 20
Length of induction (h)
wild type
uninduced
sir2
sir2 hml
hdf1
D
wild type
sir2
rad52
hdf1
sir2 rad52
time (h)
0 5 10 0 5 10 0 5 10 0 5 10 0 5 10
48
33
15
12
11
10
9
8
7

FIGURE 1- Enhanced Sensitivity of *SIR2, SIR3,* and *SIR4* mutants to *Eco*RI. (A) W303AR5 (Table 1B) cells containing plasmid-borne galactose-inducible *Eco*RI (YCpGAL:RIb) were grown in SC-URA+gal and harvested at the indicated times to test viability. Isogenic mutant derivatives of W303AR5 were treated identically to wild type. Survival was scored by colony growth on yeast extract-bactopeptone-glucose (YPD) medium, and expressed as plating efficiency normalized to the uninduced control. (B) Efficiency of transformation with *Sac*I-linearized pRS316 was determined for isogenic wild type, *hdf1, sir2, sir3, sir4, sir2 hmlα sir3 hmlα*, and *sir4 hmlα* strains. Transformation efficiency is expressed as a fraction of the efficiency in the wild type strain. Averages from at least five independent experiments are shown. (C) Isogenic strains of W303AR5 with the indicated genotypes were scored for sensitivity to *Eco*RI expression as described in (A). (D) Cells were treated as in (A) and genomic DNA was prepared (Lewis et al., 1998) from samples taken at zero, five, and ten hours and subjected to electrophoresis using a 0.5% agarose gel and stained with ethidium bromide. DNA size standards are shown in kilobase pairs (kb).

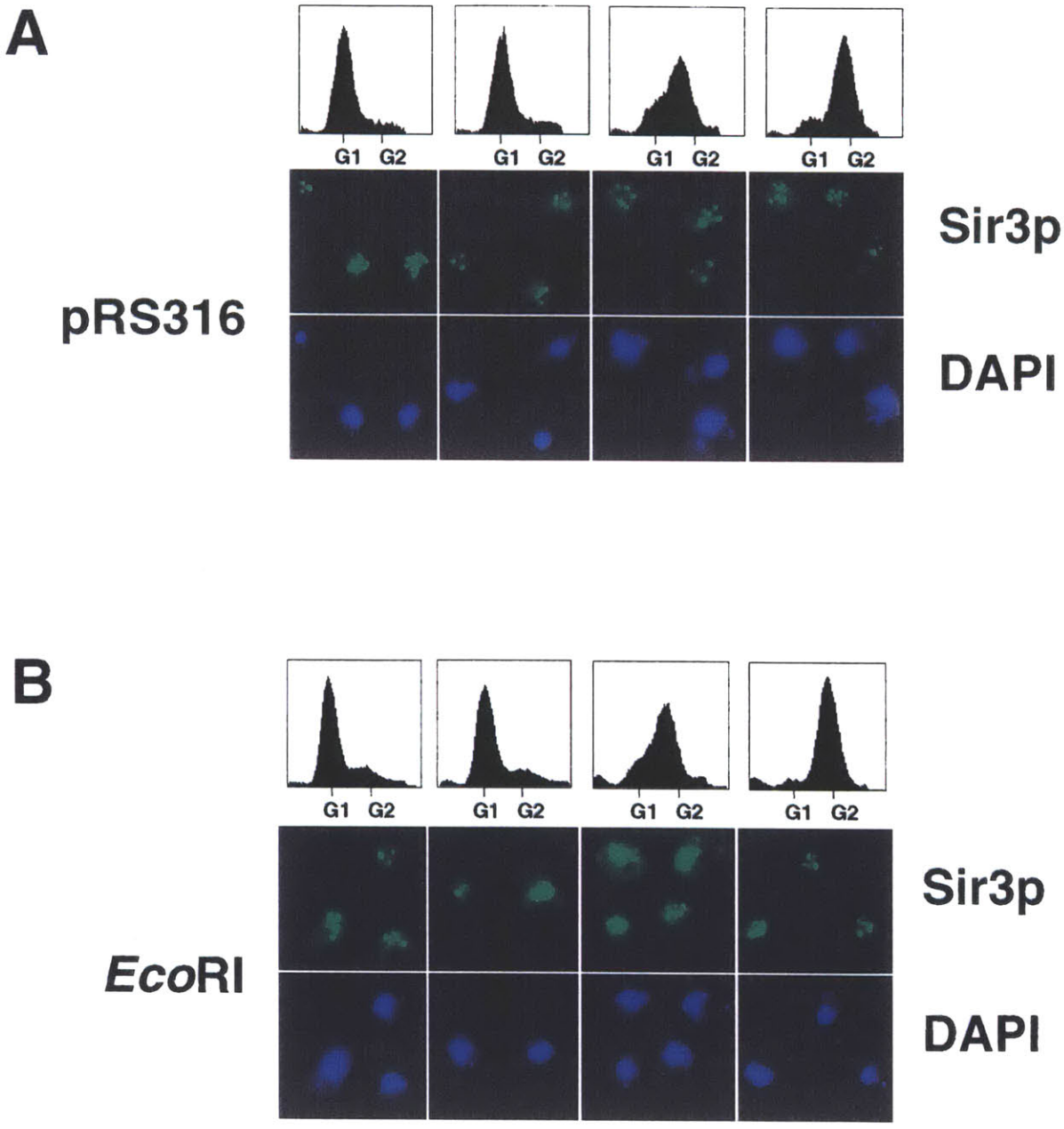
A
G1 G2
G1 G2
G1 G2
G1 G2
Sir3p
pRS316
DAPI
B
G1 G2
G1 G2
G1 G2
G1 G2
Sir3p
EcoRI
DAPI

FIGURE 2- DSBs induced by *Eco*RI result in S phase redistribution of Sir3p. (A) W303AR5 cells containing a plasmid control (pRS316) were arrested in G1 with α-factor, shifted to SC-URA+gal medium for three hours, released into YPD lacking α-factor, and samples were taken throughout the ensuing cell cycle for immunofluorescence and FACS. Cells were stained with goat fluorescein isothiocyanate (FITC)-conjugated IgG against rabbit anti-Sir3p (green) and DAPI (blue). Cell cycle progression was monitored using FACS analysis to score genomic DNA content from unreplicated (G1) to replicated (G2). (B) Cells containing plasmid-born *Eco*RI (YCpGAL:RIb) were analyzed as described in (A).

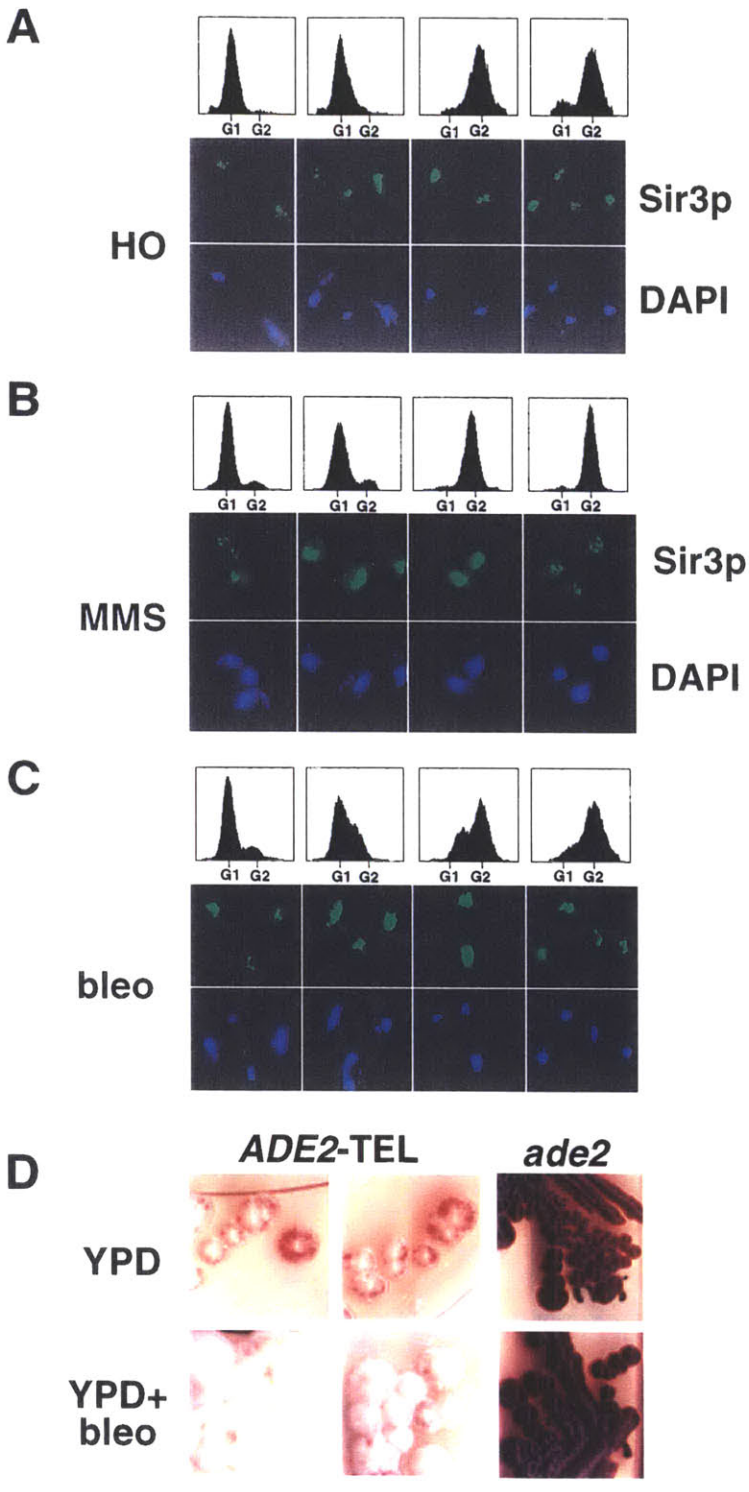
A
G1 G2
G1 G2
G1 G2
G1 G2
HO
Sir3p
DAPI
B
G1 G2
G1 G2
G1 G2
G1 G2
MMS
Sir3p
DAPI
C
G1 G2
G1 G2
G1 G2
G1 G2
bleo
D
ADE2-TEL
ade2
YPD
YPD+
bleo

FIGURE 3- Redistribution of Sir3p in response to DSBs. (A) JKM139 cells containing a galactose inducible HO endonuclease and lacking *HML* and *HMR* was grown in YPD medium, arrested in G1 by treatment with α-factor, shifted to YEP medium containing 3% galactose plus α-factor for four hours, then released into fresh YPD medium. Cell cycle and immunofluorescence analyses were performed as described in Figure 2. (B, C) W303AR5 cells were grown in YPD, arrested in G1 and treated with the indicated agent. Sir3p localization and FACS analysis were performed as described for Figure 2. (B) Cells treated with 0.02% MMS for three hours prior to release from G1 arrest. (C) Cells treated with 20 μg/ml bleomycin for two hours prior to release from G1 arrest. (D) Telomeric silencing is abrogated in the presence of bleomycin. PSY316AT (*ADE2*-TEL) or PSY316 (*ade2*) grown for four days on either YPD medium or YPD medium containing 20 μg/ml bleomycin sulfate (YPD+bleo).

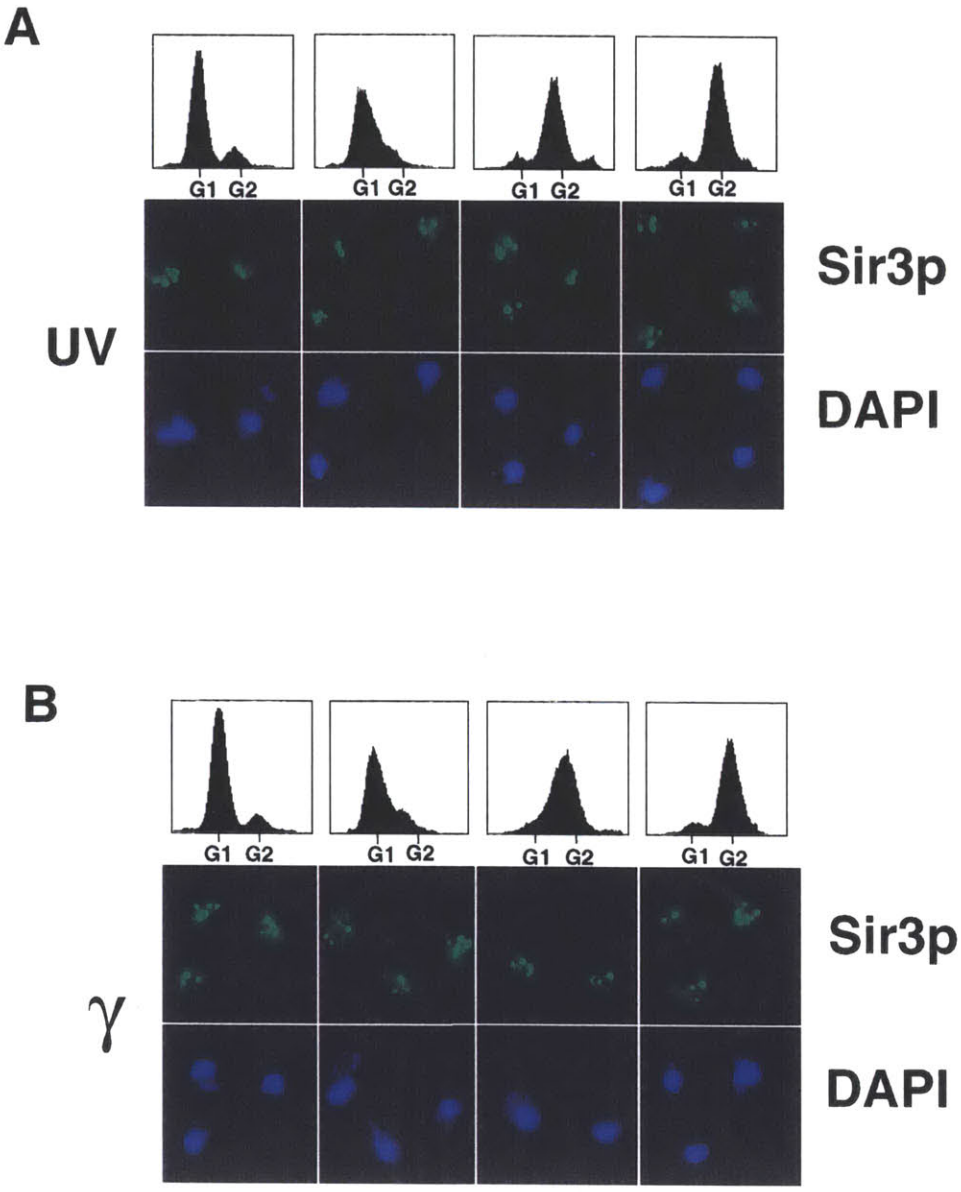
A
G1 G2
G1 G2
G1 G2
G1 G2
UV
Sir3p
DAPI
B
G1 G2
G1 G2
G1 G2
G1 G2
γ
Sir3p
DAPI

FIGURE 4- Sir3p is not released from telomeres in response to UV- or γ-irradiation. W303AR5 cells were grown in YPD, arrested in G1 and treated with the indicated agent. Sir3p localization and FACS analysis were performed as described for Figure 2. (A) Cells treated with UV irradiation (60 J/m^2) during G1 arrest. (B) Cells treated with γ-irradiation (4 Krad) during G1 arrest. Following irradiation UV or γ-treated cultures each exhibited approximately 20% survival.

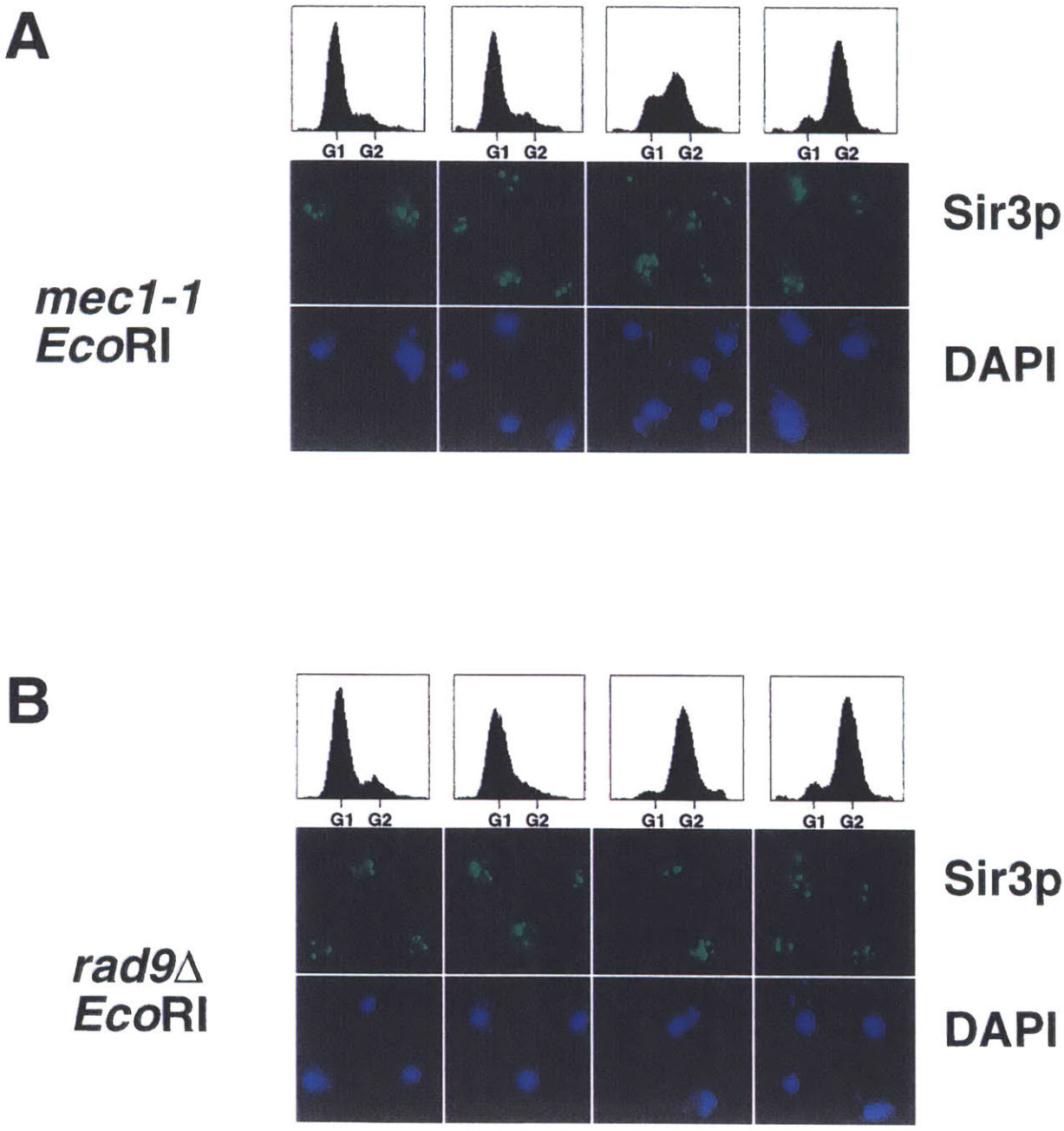
A
G1 G2
G1 G2
G1 G2
G1 G2
Sir3p
mec1-1
EcoRI
DAPI
B
G1 G2
G1 G2
G1 G2
G1 G2
Sir3p
rad9Δ
EcoRI
DAPI

FIGURE 5- Sir3p relocalization in response to DSBs requires the *MEC1* and *RAD9* checkpoint genes. Cells containing YCpGal:RIb were arrested in G1, shifted to SC-URA+gal medium for 3 hours, and cell cycle analyses were performed as previously described for Figure 2. (A) W303-1A *mec1-1* (Weinert et al., 1994). (B) W303AR5 *rad9*::*kan*R (Lee et al., 1998).

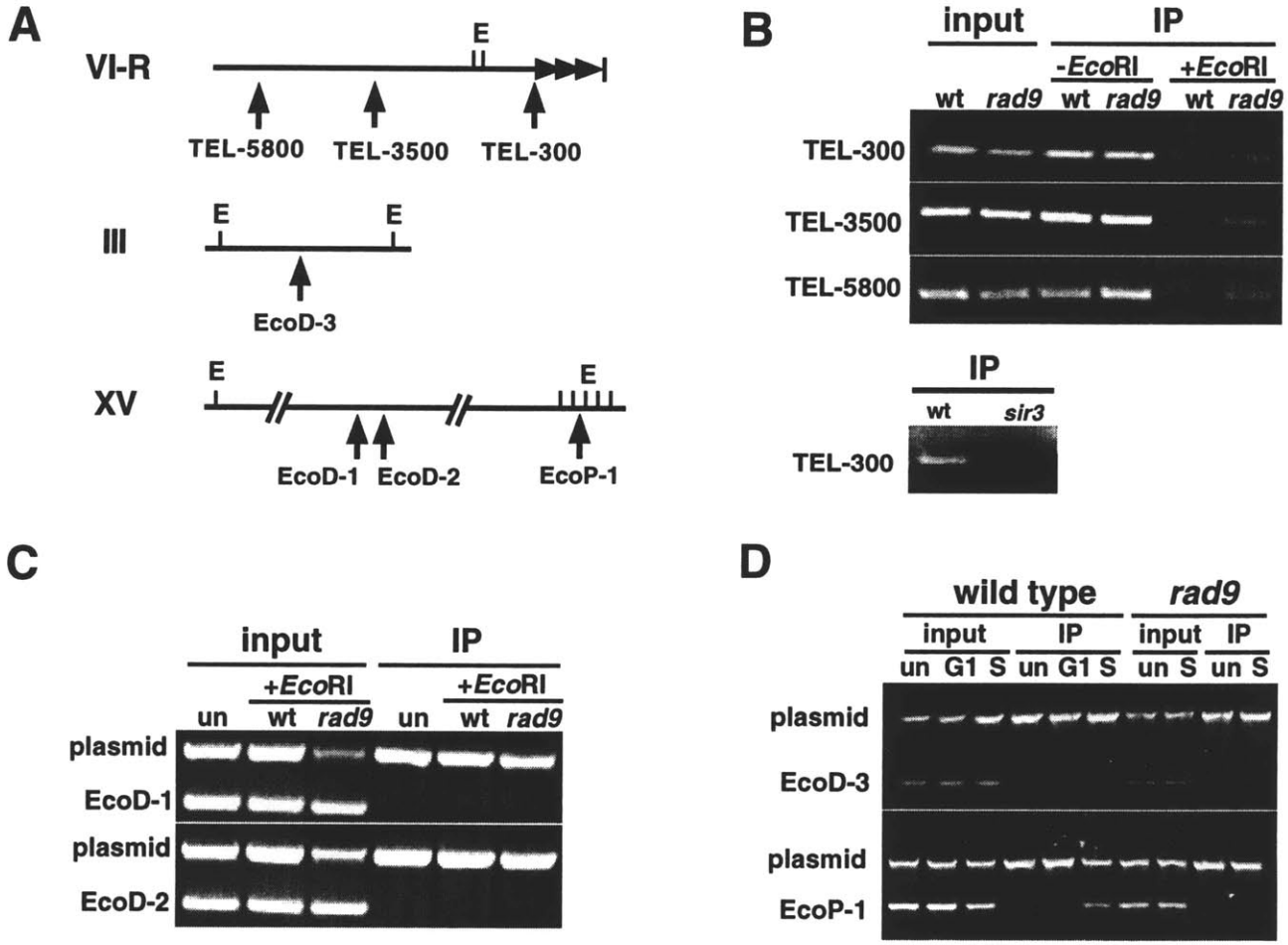
A
VI-R
E
TEL-5800
TEL-3500
TEL-300
III
E
E
EcoD-3
XV
E
E
EcoD-1
EcoD-2
EcoP-1
B
input
IP
-EcoRI
+EcoRI
wt
rad9
wt
rad9
wt
rad9
TEL-300
TEL-3500
TEL-5800
IP
wt
sir3
TEL-300
C
input
IP
+EcoRI
+EcoRI
un
wt
rad9
un
wt
rad9
plasmid
EcoD-1
plasmid
EcoD-2
D
wild type
rad9
input
IP
input
IP
un
G1
S
un
G1
S
un
S
un
S
plasmid
EcoD-3
plasmid
EcoP-1

FIGURE 6- Sir3p dissociates from telomeric DNA and associates with DSBs. DNA-protein extracts (input) were incubated with anti-Sir3p and subjected to chromatin immunoprecipitation (ChIP) as described in Materials and Methods. (A) Schematic of primer pairs used for ChIP analysis. (B) Sir3p is released from telomeric DNA at the right end of chromosome VI. W303AR5 (wt) or isogenic *rad9Δ* cells were transferred from SC-URA+glu (-*Eco*RI) or SC-URA+gal (+*Eco*RI) for four hours. Chromatin immunoprecipitates were analyzed by PCR using TEL primer pairs. (C) ChIP analysis following DSB induction using EcoD-1 and EcoD-2 primer pairs. (D) ChIP analysis of Sir3p-associated chromatin following DSB induction in G1. Immunoprecipitates analyzed using EcoD-3 and EcoP-1 primer pairs.

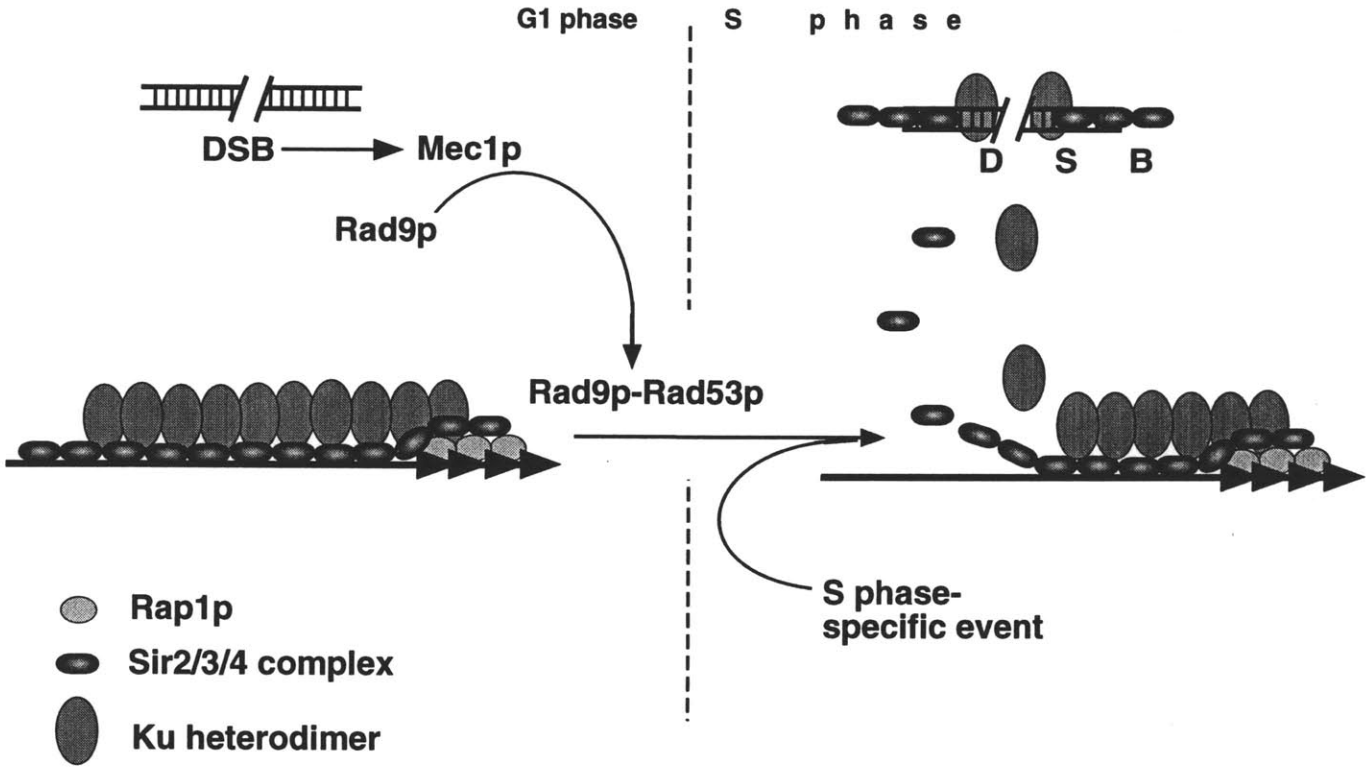
G1 phase
S p h a s e
DSB
Mec1p
Rad9p
D S B
Rad9p-Rad53p
S phase-
specific event
Rap1p
Sir2/3/4 complex
Ku heterodimer

FIGURE 7- Model for DSB induced redistribution of the Sir complex. Generation of a DSB results in a DNA damage signal involving Mec1p, Rad9p, Rad53p. This combines with a DSB-specific signal resulting in the dissolution of the telomeric Sir2/3/4p-Ku heterochromatic complex in S phase, beginning from sites more distal to the end of the chromosome. The Ku heterodimer and Sir complex interact at the DSB to promote NHEJ, perhaps by preventing inappropriate recombination, by preventing the passage of replication forks through the site, or by providing a constrained substrate for DNA ligase.

Chapter 4: The antisilencing gene *ASF1* encodes a DNA repair protein required for non-homologous end joining

The work presented in this chapter was performed in collaboration with David Sinclair

Summary

Non-homologous end joining of DNA double strand breaks (DSBs) in *Saccharomyces cerevisiae* requires the silencing genes *SIR2, SIR3,* and *SIR4.* After DSB induction during G1, Sir3p dissociates from telomeres and becomes diffuse throughout the nucleus upon entry into S phase. During this time Sir3p becomes associated with DNA near DSBs. This process requires the DNA damage checkpoint machinery, *RAD9, RAD53,* and *MEC1.* Here we show that the anti-silencing factor encoded by *ASF1,* participates in a Sir complex and is required for NHEJ. A model for the response of heterochromatin factors to DSBs is discussed.

Introduction

Transcriptional silencing in *Saccharomyces cerevisiae* is associated with telomeres, cryptic mating type (*HM*) loci, and ribosomal DNA. Marker genes placed near telomeres, *HML* or *HMR* are efficiently silenced (Gottschling et al., 1990; Aparicio et al., 1991; Aparicio et al., 1994; Abraham et al., 1984; Ivy et al., 1986). *HM* silencing is extremely efficient and epigenetically stable, while telomere-silenced genes are subject to frequent switching of transcriptional states (Gottschling et al., 1990; Aparicio et al., 1991). Silencing at telomeres and *HM* loci requires the products of *SIR2*, *SIR3*, and *SIR4* (Ivy et al., 1986; Aparicio et al 1991). Mutation of any one of these genes causes a complete loss of silencing. In haploid cells this results in simultaneous expression of mating type **a** and mating type α information, and inability to mate. Sir2p, Sir3p, and Sir4p participate in a complex that interacts with a variety of other factors, including Rap1p (Kyrion et al., 1993; Sussel and Shore, 1991) and Histones H3 and H4 (Hecht et al., 1995; Johnson et al., 1990), to form transcriptionally repressive heterochromatin. Silent heterochromatin at telomeres also requires Hdf1p, Hdf2p, and Cdc13p, and is important for telomere maintenance as well as silencing (Laroche et al., 1998; Boulton and Jackson 1998; Nugent et al., 1998; Nugent et al., 1996).

Telomeric heterochromatin is very dynamic. The transcriptional state near telomeres can switch between active and inactive, and the new state is heritable for several generations before the next switch (Gottschling et al., 1990). Under normal conditions, silent heterochromatin spreads from the telomere approximately 5 kilobase pairs (kb) inward toward the centromere (Venditti et al., 1999; Vega-Palas et al, 1998; Strahl-Bolsinger et al., 1997;

Hecht et al., 1996), but can extend as far as 25 kb when Sir3p is overexpressed (Renauld et al., 1993). Sir3p is a phosphoprotein that becomes hyperphosphorylated by the mitogen-activated protein (MAP) kinase pathway in response to mating pheromone, heat shock, or starvation, resulting in increased telomere silencing (Stone and Pillus, 1996). Telomeres also regulate silencing at other regions of the genome by sequestering silencing factors via interaction with Rap1 (Smith et al., 1998; Marcand et al., 1996). Release from telomere sequestration results in decreased telomere silencing but increased silencing at non-telomeric loci.

Ensuring the integrity of genomic DNA is crucial to all cells. Yeast cells can efficiently repair DSBs by two mechanisms: homologous recombination and non-homologous end joining (NHEJ). Non-homologous end joining requires the Ku70/Ku80 DNA end binding dimer (encoded by *HDF1* and *HDF2* respectively) (Tsukamoto et al., 1996; Milne et al., 1996; Boulton and Jackson, 1996), the Xrs2p/Rad50p/Mre11p complex (Tsukamoto et al., 1997; Milne et al., 1996; Moore and Haber, 1996), Sir2p/3p/4p (Tsukamoto et al., 1997), and DNA ligase IV (*DNL4*) (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997). *SIR2*, *SIR3*, and *SIR4* are required for the efficient recircularization of linear plasmid DNA by NHEJ and for the survival of DSBs incurred in chromosomal DNA (Mills et al., 1999; Tsukamoto et al., 1997). Following induction of chromosomal DSBs Sir3p is released from telomeres and becomes diffuse throughout the nucleus (Martin et al., 1999; Mills et al., 1999). Release of Sir3p from telomeres requires the DNA damage checkpoint machinery and entry into the S phase of the cell cycle. Mutation of *RAD9*, *RAD53* or *MEC1* results in inability to redistribute Sir3 in response to DNA damage. Sir3p then

becomes associated with DNA near DSBs, perhaps to participate in Ku-dependent repair (Martin et al., 1999; Mills et al., 1999).

To identify additional components of telomeric heterochromatin, a screen for high copy antagonists of telomere silencing was performed. One strong high-copy silencing antagonist identified in this screen was the anti-silencing factor gene *ASF1*. *ASF1* was previously identified in screens for high copy disruptors of telomere or *HM* silencing (Le et al., 1997; Singer et al., 1998). *ASF1* transcription is cell cycle regulated, reaching peak levels during S-phase. Mutation of *ASF1* results in sensitivity to the DNA alkylating agent methyl methane sulfonate (MMS) and causes a slight defect in minichromosome maintenance. Here we demonstrate that *ASF1* is required for NHEJ, and may assist in recruitment of the Sir complex to DSBs.

RESULTS

ASF1 summary

ASF1 was identified in the screen for high copy disruptors of telomere silencing described in Chapter 2. Briefly, a telomere silencing reporter strain was constructed by integrating *ADE2* near the telomere on the right arm of chromosome V and *URA3* near the telomere on the left arm of chromosome VII. In this strain (PSY316AUT) silencing of the *ADE2* gene produces colonies with red sectors, and silencing of *URA3* results in resistance to the drug 5-fluoroorotic acid (5-FOA). PSY316AUT was transformed with a 2μ yeast genomic library and 80,000 transformants were screened for derepression of the telomeric *ADE2* and *URA3* genes. *ASF1*

was isolated three times as a strong antagonist of telomere silencing. The cloning of *ASF1* and the characterization of its silencing phenotypes are detailed in Chapter 2 and summarized below.

ASF1 encodes a 30 kD protein with a highy acidic C-terminus (Figure 1A). The N-terminal half of the protein is evolutionarily conserved from yeast to humans (Figure 1B). However, no function has yet been assigned to *ASF1* homologues in any species except *Saccharomyces*.

The effects of overexpression or mutation of *ASF1* on transcriptional silencing were determined. Introduction of high copy *ASF1* resulted in strong derepression of telomeric marker genes. High copy *ASF1* also depressed *ADE2* silenced by an *HMR* silencer or *ADE2* integrated into rDNA. Thus, when overexpressed, *ASF1* disrupts silencing at all known silent loci. In contrast, disruption of *ASF1* resulted in a strong increase in telomere silencing, and no effect on rDNA silencing. These findings suggested that *ASF1* may encode a telomere specific anti-silencing factor that can antagonize silencing throughout the genome when overexpressed.

Asf1p is a nuclear protein

To further characterize *ASF1* we developed a polyclonal antibody against the full length protein. Immunizing antigen was used to affinity purify anti-Asf1p antibody from chicken immune serum. Affinity purifed anti-Asf1p antibody specifically recognized both recombinant Asf1p and Asf1p from yeast whole cell extracts (WCE) by Western blot analysis (Figure 2A). No bands are observed by Western blot of WCE derived from an *asf1*Δ strain.

This affinity purified anti-ASF1 antibody was used to determine the subcellular localization of Asf1p by indirect immunofluorescence. Wild type or *asf1*Δ cells were grown to mid-log phase, fixed, and stained with anti-Asf1p antibody and the DNA binding dye, DAPI. In wild type cells, Asf1p was concentrated in the nucleus and showed a diffuse staining pattern with no detectable sub-nuclear localization (Figure 2B). Fluorescent signal above background was not detected in fixed *asf1*Δ cells (Figure 2C).

ASF1 is required for NHEJ

Mutation of *ASF1* results in sensitivity to MMS (Le, et al., 1997) suggesting that Asf1p is involved in DNA repair. Therefore, the role of *ASF1* in NHEJ was tested using a linear plasmid transformation assay. Survival following transformation requires recircularization of the linear plasmid by NHEJ. Transformation efficiency is expressed as colony forming units (CFU) after transformation with linear plasmid divided by CFU with circular plasmid and all results are shown normalized to wild type. Mutation of *ASF1* resulted in approximately a 12-fold reduction in transformation efficiency relative to wild type (Figure 3A). The transformation efficiency in the *asf1*Δ strain is similar to that observed in *hdf1*Δ or *sir2*Δ strains, which are shown for comparison. By contrast, mutation of *RAD52*, required for homolgous recombination, did not decrease plasmid NHEJ efficiency. This suggests that the DNA repair defect in *asf1*Δ strains is in NHEJ.

Because the DNA damage responsive cell cycle checkpoint pathway is required for release of NHEJ factors from telomeres (Mills et al., 1999; Martin et al., 1999), we predicted that the checkpoint machinery would also

be required for NHEJ of plasmid DNA. Plasmid end-joining assays were performed, as described, in strains mutant for *RAD53, RAD9,* or *MEC1*. Wild type and *hdf1*Δ strains were included as controls. Surprisingly, all three checkpoint mutants exhibited only a 2-fold decrease in NHEJ efficiency relative to wild type (Figure 3B). By comparison, *hdf1*Δ cells show a 20-fold reduction in NHEJ efficiency from wild type.

ASF1 mutants are sensitive to DNA damaging agents

Expression of the restriction endonuclease *EcoR*I *in vivo* in *S. cerevisiae* results in DSBs in the genome (Barnes and Rine, 1985; Lewis et al., 1998). NHEJ is required for repair of *EcoR*I induced DSBs and resistance to *EcoR*I expression. To test the involvement of *ASF1* in the repair of chromosomal DSBs, wild type, *asf1*Δ, and *hdf1*Δ cells were compared for sensitivity to *EcoR*I expression (Figure 4). *EcoR*I, under the control of a galactose inducible promoter, was expressed for the indicated times and cells were plated to YPD to assay CFU. Survival is shown as the percentage of cells plated that formed colonies. Consistent with previous results, wild type cells were very resistant to *EcoR*I induced DSBs, showing little loss of colony forming potential even after 20 hours of *EcoR*I expression. Also consistent with previously published results, *hdf1*Δ cells which are defective in NHEJ, were very sensitive to *EcoR*I, showing a 10-fold reduction in colony forming potential after 20 hours of *EcoR*I expression. Similarly, *asf1*Δ cells were hypersensitive to expression of *EcoR*I, displaying colony forming potential comparable to that of the *hdf1*Δ strain at each time point.

Since *ASF1* is required for resistance to MMS, the sensitivity of *asf1*Δ cells to a variety of other DNA damaging agents was also tested. Cultures of *asf1*Δ and control strains were plated in a 10-fold dilution series on YPD medium or on YPD medium containing either MMS or the DSB inducing agent bleomycin. In agreement with published results, *asf1*Δ cells were hypersensitive to 0.005% MMS(Figure 5A). Consistent with a role in NHEJ, *asf1*Δ cells were also hypersensitive to 2 μg/ml bleomycin (Figure 5B).

We wished to determine if the DNA repair defect in *asf1*Δ strains affected the repair of lesions that are not addressed by NHEJ. Cultures of *asf1*Δ and control strains were UV-irradiated and plated in a 10-fold silution series on YPD to assay survival. Unirradiated samples of each strain were treated identically and equivalently plated (Figure 6A). Mutation of *asf1*Δ did not result in pronounced sensitivity to UV-irradiation. Wild type, *sir2*Δ, *sir2*Δ *hml*Δ, and *rad9*Δ strains are shown as controls. Likewise, *asf1*Δ strains are as resistant to γ-irradiation as wild type (Figure 6B), while *sir2*Δ cells are slightly sensitive and *rad9*Δ cells are hypersensitive. This is consistent with a role for *ASF1* in the processing of lesions that specifically require NHEJ for repair.

ASF1 does not affect release of Sir3 from telomeres after DNA damage

Wild type cells treated with a DSB inducing agent while arrested in the G1 phase of the cell cycle show Sir3p redistribution throughout the nucleus upon entry in to S phase (Mills et al., 1999). To determine if *asf1*Δ cells are defective in Sir3p release, wild type and *asf1*Δ strains were

compared for the ability to redistribute Sir3p following induction of DSBs. Cells were arrested in G1 by treatment with α-mating pheromone, treated with bleomycin while arrested, and processed for indirect immunofluorescence at intervals preceding or following release into fresh medium. Normal telomeric Sir3p staining is observed in untreated *asf1*Δ cells (data not shown). Interestingly, mutation of *ASF1* does not result in observable differences from wild type in the ability to release Sir3p (Figure 7). This suggests that the NHEJ defect and DSB hypersensitivity in *asf1*Δ does not result from a failure to release NHEJ factors from sequestration at telomeres.

Asf1p interacts with Sir2p

Asf1p may modulate silencing and DSB repair by directly interacting with members of the Sir complex. To test this possibility, the interaction between Asf1p and Sir2p was probed by immunoprecipitation analysis. Immunoprecipitates were prepared from whole cell extracts of wild type, *asf1*Δ, or *sir2*Δ strains using either chicken anti-Asf1p antibody or rabbit anti-Sir2p antibody (gift of C. Armstrong). All immunoprecipitates were then probed, by Western blot, for Asf1p (Figure 8A). Input whole cell extract clearly showed Asf1p in wild type and *sir2*Δ, but not *asf1*Δ, lanes (Figure 8A, top panel). Asf1p was efficiently immunoprecipitated from wild type and *sir2*Δ with anti-Asf1p antibody (Figure 8A, middle panel). Asf1p was also precipitated with anti-Sir2p antibody in wild type, but not in either *asf1*Δ or *sir2*Δ (Figure 8A, bottom panel). This indicates that Sir2p was present in Asf1p immunoprecipitates, and that precipitation of Sir2p with the anti-Asf1p antibody required the presence of Asf1p. To further verify this

interaction immunoprecipitation was preformed from a strain containing Sir2p tagged at its C-terminus with a triple HA-epitope. Immunprecipitation was carried out using a monoclonal anti-HA antibody (12CA5, Babco) and precipitates were probed for Sir2p-HA or Asf1p (Figure 8B). Sir2p-HA was efficiently and specifically immunoprecipitated with the anti-HA antibody (Figure 8B, top panel), and Asf1p was also present in this immunoprecipitate (Figure 8B, bottom panel). Finally, anti-Asf1p antibody was used to prepare immunoprecipitates from the Sir2p-HA strain (Figure 8C). Control precipitates were prepared using protein-A sepharose but no antibody, or using chicken pre-immune serum. Immunoprecipitates were probed by Western blot with anti-HA antibody, and Sir2p-HA was detected only in precipitates using Asf1p-specific antibody. These results demonstrate that Sir2p and Asf1p can efficiently and specifically be co-immunoprecipitated, suggesting that they interact in a complex.

DISCUSSION

In *Saccharomyces cerevisiae* two efficient modes of DNA repair address DNA double strand breaks: homologous recombination and non-homologous end joining. NHEJ requires the homologues of the mammalian DNA end binding factors, Ku70 and Ku80 (Tsukamoto et al., 1996; Milne et al., 1996; Boulton and Jackson, 1996). In addition NHEJ requires *XRS2, RAD50, MRE11* (Tsukamoto et al., 1997; Milne et al., 1996; Moore and Haber 1996), the Sir2p/3p/4p complex (Tsukamoto et al, 1997) and DNA ligase IV (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997), suggesting that end processing and chromatin formation are important

aspects of DSB repair. It was previously shown that Sir3p is released from telomeres and distributes throughout the nucleus in response to DSBs (Mills et al., 1999; Martin et al., 1999). This release requires a functional DNA damage checkpoint and entry into S phase (Mills et al., 1999). Here we show that *ASF1*, encoding an antisilencing factor, is also required for NHEJ.

ASF1 modulates telomere silencing

ASF1 was initially identified in screens for high copy anti-silencing activity (Le et al., 1997; Singer et al., 1998). Overexpression of *ASF1* severely reduces the strength of silencing at *HM* loci, telomeres and rDNA, while disruption of *ASF1* results in an increase in silencing at telomeres (Chapter 2). These findings suggested that *ASF1* might encode a telomere specific antisilencing factor. Overexpression of *ASF1* could globally compromise silencing by disrupting silencing complexes, by recruiting silencing factors away from their normal sites activity, or by exerting normal anti-silencing activity throughout the genome. Asf1p is a small molecular weight protein with numerous acidic residues throughout its C-terminal half. Acidic regions of many proteins are known to have transcriptional activation activity in yeast (reviewed in Struhl, 1995). Therefore, Asf1p may act as a telomere specific anti-silencing factor by creating a more transcriptionally activated environment via its acidic C-terminus. A telomere specific anti-silencing factor might prevent excessive spreading of telomeric heterochromatin or inappropriate silencing of telomere proximal genes. *ASF1* transcription is also cell cycle regulated, reaching peak transcript levels during S phase. Since silencing is known to require passage through S phase for establishment (Triolo and Sternglanz, 1996), the S phase

function of *ASF1* may be involved in the establishment of silent heterochromatin.

However, it is likely that *ASF1* has functions in addition the its anti-silencing activity. Indirect immunofluorescence demonstrated that Asf1p is distributed throughout the nucleus. This suggests, at least, that the telomere anti-silencing activity of *ASF1* is not mediated by concentration of the protein near telomeres. Deletion of *ASF1* also results in a slight slow growth phenotype and lowers the restrictive temperature of a *CDC13* mutant strain (Le et al., 1997). It is not clear if these phenotypes are related to the telomere silencing phenotype in *asf1*Δ cells or if *ASF1* has other functions, perhaps including a role in DNA synthesis. Defects in DNA synthesis, or other S phase processes such as checkpoint control, could account for the slow growth of *asf1*Δ cells.

Asf1p is a non-homologous end joining factor

In addition to silencing and slow growth phenotypes, mutation of *ASF1* results in a slight decrease of minichromosome maintenance and sensitivity to the DNA alkylating agent MMS. The major lesion induced by MMS is 3-methly adenine, but DSBs can also be generated (Schwartz, 1989; Mitchel and Morrison, 1987). To determine if the DNA repair defect in *asf1*Δ strains is in NHEJ, plasmid end joining and sensitivity to other DSB-inducing mutagens were tested.

Reciricularization of linear plasmid DNA introduced by transformation requires NHEJ machinery (Boulton and Jackson, 1996; Tsukamoto et al., 1996; Milne et al., 1996), and occurs very inefficiently by other DNA repair pathways. Therefore, a plasmid repair assay is a sensitive

test for involvement of a gene in NHEJ. Consistent with a role in NHEJ, mutation of *ASF1,* results in a 12-fold reduction in linear plasmid repair efficiency relative to wild type. This is approximately the same magnitude reduction in repair efficiency observed in strains mutant for the end-joining factor Ku70 (Tsukamoto et al., 1996; Milne et al., 1996). By contrast, cells mutant for the homologous recombination gene *RAD52* recircularize plasmid DNA as efficiently as wild type. This demonstrates that *ASF1* is required for NHEJ repair of DSBs.

ASF1 is also required for resistance to DSBs generated in genomic DNA *in vivo,* since strains mutant for *ASF1* are hypersensitive to *EcoR*I expression, MMS, and bleomycin. This sensitivity is not due to general inability to repair damaged DNA because *asf1*Δ cells are resistant to UV- or γ-irradiation, which induce lesions that are not repaired by NHEJ. Therefore, *asf1*Δ cells are specifically sensitive to those DNA lesions that require NHEJ for repair. Together with the plasmid DSB repair, these findings demonstrate that *ASF1* encodes a NHEJ factor required for repair of DSBs *in vivo.*

Role of Asf1p in NHEJ

Since *ASF1* influences telomere silencing and is required for efficient NHEJ we speculated that mutation of *ASF1* would interfere with normal release of Sir3p following induction of DSBs. Surprisingly, *asf1*Δ cells still exhibit redistribution of Sir3p during S phase, following treatment with bleomycin during G1. Therefore the NEHJ defect in an *asf1*Δ cannot be due to inability to release Sir3p from telomeres.

Although Sir3p redistribution requires *RAD9, RAD53,* and *MEC1* (Mills et al., 1999; Martin et al., 1999), mutation of any one of these three checkpoint genes only results in a 2-fold reduction in plasmid end-joining efficiency. This suggests that cells possess a backup mechanism capable of carrying out non-homologous end joining in the absence of Sir3p release.

We propose a model in which Asf1p acts downstream of the checkpoint machinery in responding to DSBs (Figure 9). DSBs are sensed by the checkpoint machinery, which transduces a signal to telomeres resulting in the release of telomere bound repair proteins, including the Sir complex, during S phase. However, we postulate that there is a small pool of Sir proteins, and perhaps other DNA repair factors, that do not normally reside at telomeres. Recruitment of this pool does not require Rad9p/Rad53p/Mec1p dependent signaling for release and accounts for the residual NHEJ seen in checkpoint mutant strains. Both this pool, and the telomeric pool of repair factors require Asf1p for subsequent steps in NHEJ. This model is further supported by the finding that Asf1p can be found in a complex with Sir2p. It is not known yet if the interaction between Asf1p and Sir2p is direct or if other factors are also present in the same complex. Asf1p may be required for priming the Sir complex for association with DSBs or may be required to directly recruit the complex to sites of DNA damage. These possibilities are currently being investigated.

Implications for DNA repair in other eukaryotes

Nonhomologous end joining is important in vertebrates as well as yeast. In vertebrates NHEJ is utilized in certain developmental processes, in addition to repair of damaged DNA. Ku70, Ku80, DNA-PK_{cs}, and DNA

ligase IV are all required for repair of chromosomal DSBs introduced during maturation of antigen receptor genes (Gao et al., 1998; Gu et al., 1997; Ouyang et al 1997). Mutation of these NHEJ factors leads to severe combined immune deficiencies or embryonic lethality, as well as hypersensitivity to ionizing radiation and DSB repair defects (Barnes et al., 1998; Gao et al., 1998; Frank et al., 1998; Gu et al., 1997; Ouyang et al., 1997). Moreover, mutation of murine DNA ligase IV or the ligase IV associated factor XRCC4 results in extensive neuronal apoptosis in the embryonic brain (Gao et al., 1998), suggesting that NHEJ factors may play other roles in development as well.

ASF1 homologues have been identified in *S. pombe, C. elegans,* mouse, and human possibly suggesting that its function in NHEJ has been evolutionarily conserved. The conservation of *ASF1*, as well as *SIR2*, between yeast and mammals hints that chromatin structure and reorganization may also play a role in NHEJ in higher eukaryotes. Understanding the nature of this chromatin and its involvement in DNA repair could have important implications in the understanding of human diseases that arise from defective DNA repair, including many types of cancer.

MATERIALS AND METHODS

Yeast Strains and Plasmids

All yeast strains in this study are derivatives of W303AR5, unless otherwise indicated. *ASF1* was disrupted using plasmid 119t-2 as described in Chapter 2. All other yeast strains are described in Chapter 3, Table 1A.

*EcoR*I was expressed from the plasmid YCpGAL:RIb, containing the *URA3* selectable marker gene and *EcoR*I controlled by a galactose-inducible promoter. YCpGAL:RIb was maintained in yeast cells by selection in synthetic complete yeast medium lacking uridine (SC-URA) and containing 2% glucose (SC-URA+glu). *EcoR*I expression was induced by transferring cells from from SC-URA+glu to SC-URA medium contatining 3% galactose as the sole carbon source (SC-URA+gal).

NHEJ and Survival assays

Non-homologous end joining was assayed by the linear plasmid transformation assay method. Strains to be assayed were cultured overnight at 30°C in yeast extract/peptone/2% glucose (YPD) medium, diluted 1:50 into fresh YPD medium for 3-4 hours, and transformed using a standard lithium actetate transformation procedure. For each strain, parallel tranformations were performed using either 150 ng of circular pRS316 plasmid, or 450 ng of pRS316 linearized within the polylinker using *Sac*I restriction endonuclease. Transformation efficiency is expressed as CFU with linear plasmid divided by CFU with circular plasmid. All results are shown normalized to the transformation efficiency of the wild type strain.

Sensitivity to *EcoR*Iexpression was determined by culturing cells containing the plasmid YCpGAL:RIb over night in SC-URA+glu medium at 30°C to maintain the plasmid. Cells were diluted into fresh SC-URA+glu medium and cultured 4 hours at 30°C. *EcoR*I was induced by transfering cells to SC-URA+gal medium. After 0, 5, 10, or 20 hours of *EcoR*I induction cell tuter was determined and cells were plated to YPD medium to assay CFU. Survival is expressed as the percent of cells plated that form

colonies. Sensitivity to γ-irradiation was determined by culturing cells to mid-log phase in YPD medium, irradiating with the indicated dose, plating to YPD and determining survival as with *EcoR*I. Sensitivity to MMS or bleomycin was determined by10-fold serially diluting YPD cultures of the indicated strain and spotting to YPD or YPD containing 0.005% MMS or 2μg/ml bleomycin. Sensitivity to UV irradiation was determined by spotting 10-fold serially diluted cultures that were either untreated or damaged with 60 J/m^2 UV-irradiation.

Immunofluorsecence

Cell fixation, spheroplasting, and indirect immunofluorescence were performed as described (Chapter 3). Indirect immunofluorescence to localize Asf1p was performed using affinity purified chicken polyclonal antibodies directed against full length Asf1p. Sensitivity and specificity of this antibody were tested by Western blot analysis using yeast whole cell extract and recombinant full length Asf1p expressed and purified from *E. coli*. Fixed spheroplasts were adhered to glass microscope slides and incubated overnight at 37°C with affinity purified anti-Asf1p antibody. Slides were washed as described and incubated two hours at 37°C with biotinylated goat anti-chicken antibody. Finally slides were stained with FITC-avidin and processed as described.

Co-immunoprecipitation

Yeast whole cell extract (WCE) for co-immunoprecipitation was prepared from YPD cultures grown to OD_{600}=1.0 to 1.3. Cells were harvested and resuspended in 500 μl IP Lysis Buffer (20mM HEPES pH 7.4;

100mM potassium acetate; 2mM magnesium actetate; 6mM phenylmethylsulfonyl fluoride). Cell suspensions were vortexed with 300 µl glass beads (0.5 mm diameter) for 7 mintues at full speed on a Vortex Genie vortexer at 4°C. For each immunoprecipitation reaction 500 µg WCE was brought to a final volume of 500 µl with cold IP Reaction Buffer (20mM HEPES pH 7.4; 10% glycerol; 12.5 mM magnesium chloride; 0.1mM EDTA; 0.2% NP-40; 100mM potassium acetate). Extract+IP Reaction Buffer was incubated one hour at 4°C with 20 µl immune serum against, and micro-centrifuged 1 minute at maximum velocity to pellet aggragated material. Supernatant was incubated one hour with 20 µl Protein A-conjugated sepharose and gently pelleted by micro-centrifugation. Sepharose pellets were washed twice with 1 ml cold IP Reaction Buffer then twice with 1 ml cold IP Reaction Buffer with 0.5 mg/ml Bovine Serum Albumin (fraction V). Pellets were resuspended in 20-35 µl protein sample loading buffer with bromophenol blue, boiled for three minutes, and loaded onto 4-15% gradient polyacrylamide mini-gels for Western blot analysis by standard methods.

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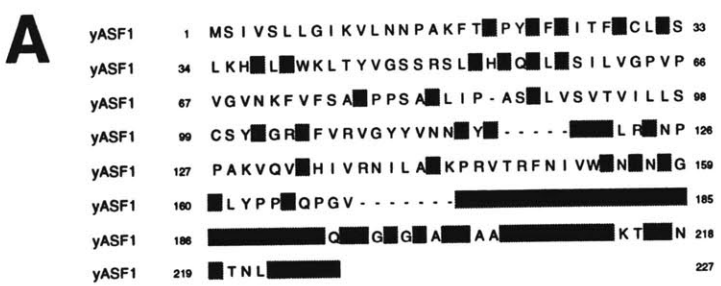
A
yASF1 1 MSIVSLLGIKVLNNPAKFT PY F ITF CL S 33
yASF1 34 LKH L WKLTYVGSSRSL H Q L SILVGPVP 66
yASF1 67 VGVNKFVFSA PPSA LIP-AS LVSVTVILLS 98
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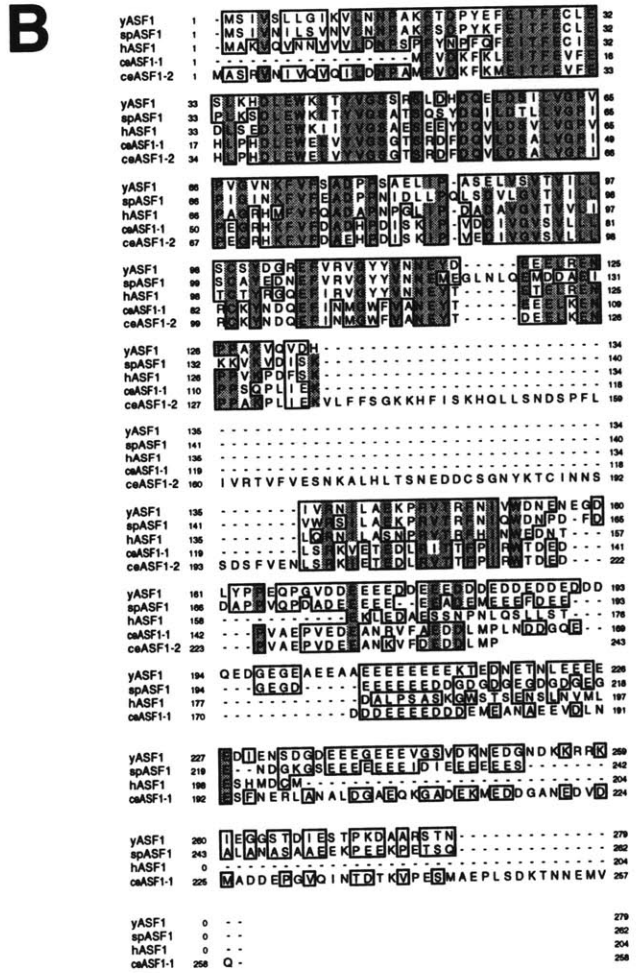

B
yASF1
spASF1
hASF1
ceASF1-1
ceASF1-2

FIGURE 1- Sequence features of the protein encoded by *ASF1*. (A). Amino acid sequence of Asf1p with acidic residues shaded. (B). Multiple sequence alignment of Asf1p homologues from *Saccharomyces cerevisiae* (yASF1), *Schizosaccharomyces pombe* (spASF1), human, (hASF1), and *Caenorhabditis elegans* (ceASF1-1 and ceASF1-2). Similar amino acids are boxed and identical amino acids are shaded.

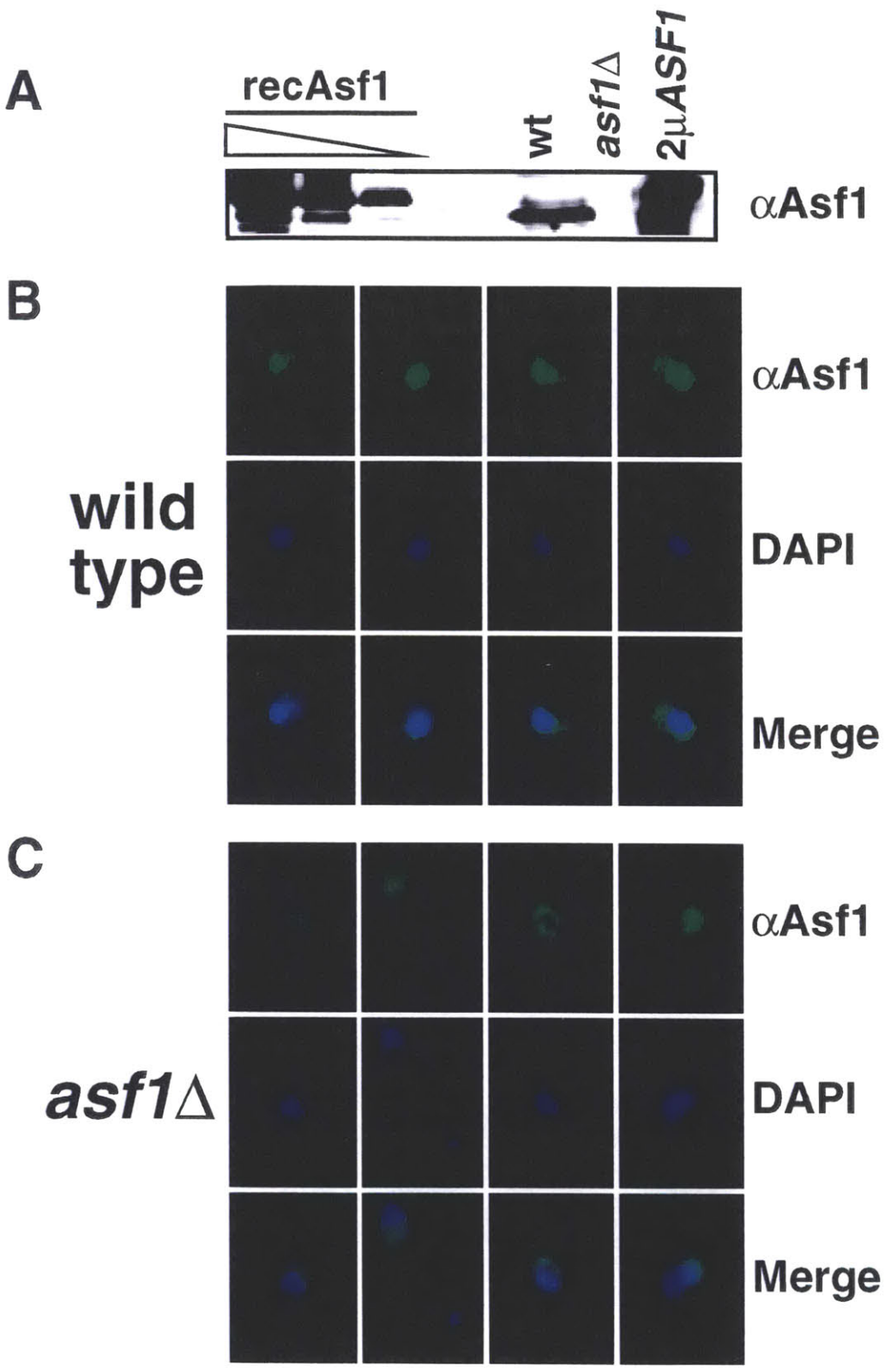
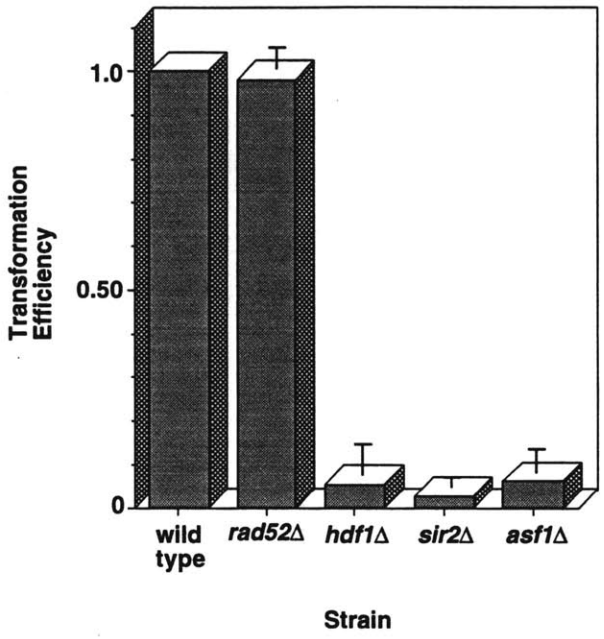

A
recAsf1
wt
asf1Δ
2μASF1
αAsf1
B
wild type
αAsf1
DAPI
Merge
C
asf1Δ
αAsf1
DAPI
Merge

FIGURE 2- Immunolocalization of Asf1p. (A). Western blot probed with affinity purified anti-Asf1p antibody from chicken immune serum. First three lanes were loaded with 500ng, 100ng, and 20ng respectively of the recombinant, full length Asf1p used for immunization. Subsequent lanes were loaded with 15μg whole cell extract from yeast strains with the indicated genotype. wt=wildtype; *asf1*Δ= Tn1000::*HIS3* dirsuption of *ASF1*; 2μ*ASF1*= wildtype containing a 2μplasmid with *ASF1* transcribed from its native promoter. (B). Indirect immunofluorescence to detect Asf1p. Each column shows staining for a single cell. Wildtype W303AR5 cells were fixed, spheroplasted, and stained with the anti-Asf1p antibody used in Figure 1A. The anti-Asf1p antibody was detected using biotinylated anti-chicken antibody, followed by Fluoroscein isothiocyanate (FITC)-conjugated avidin (shown in green). DNA was stained using DAPI (shown in blue). Merge represents the overlap of the FITC and DAPI signals. (C). W303AR5 cells containing a disruption of *ASF1* were treated as described for Figure 1B. Each column shows staining for a single cell.

A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0.50
0
Transformation Efficiency
wild type
rad52Δ
hdf1Δ
sir2Δ
asf1Δ
Strain

B

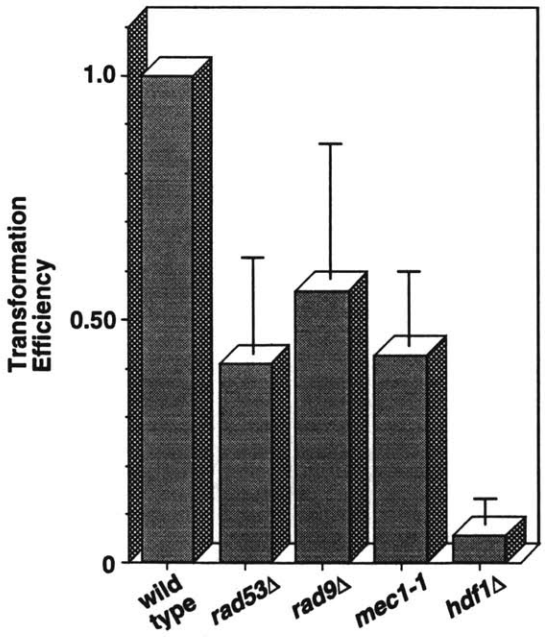

1.0
0.50
0
Transformation Efficiency
wild type
rad53Δ
rad9Δ
mec1-1
hdf1Δ

FIGURE 3- Plasmid nonhomologous end-joining assays. Tranformation-based plasmid repair assays were performed as described. Each column represents the average of at least three independent transformations. Transformation efficiency is represented as the number of colonies following tranformation with linear plasmid divided by number of colonies following tranformation with circular plasmid. All efficiencies are normalized to wild type. (A). The efficiency of NHEJ in *asf1*Δ compared with the efficiency in *rad52*Δ, *hdf1*Δ, and *sir2*Δ. (B). Plasmid NHEJ assays performed in the DNA damage checkpoint mutants *rad53*Δ, *rad9*Δ, and *mec1-1*. NHEJ efficiency in *hdf1*Δ is shown for comparison.

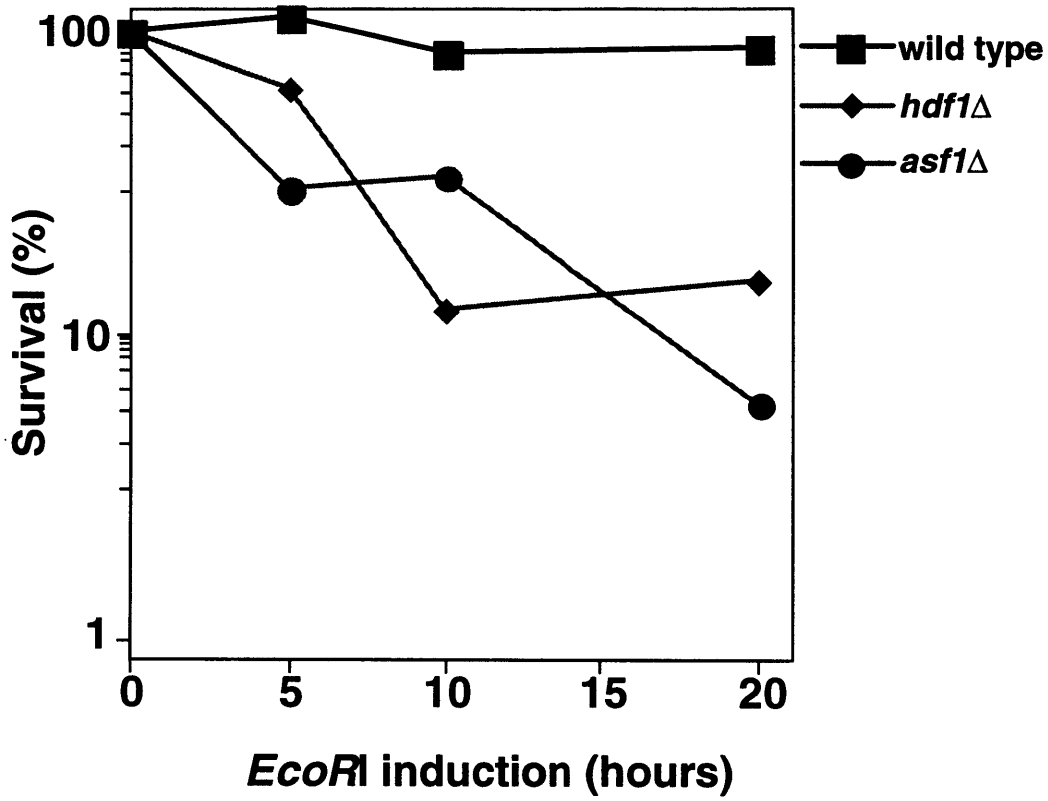
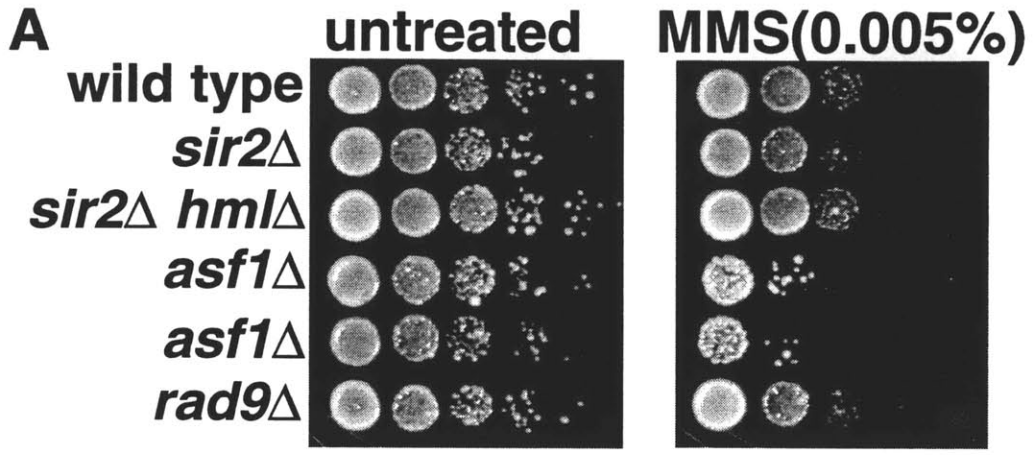

100
10
1
Survival (%)
0
5
10
15
20
EcoRI induction (hours)
wild type
hdf1Δ
asf1Δ

FIGURE 4- Sensitivity of *ASF1* mutant cells to *EcoR*I expression. Wildtype, *hdf1*Δ, and *asf1*Δ strains were grown in medium containing glucose, shifted to galactose to induce *EcoR*I for the indicated length of time, and plated onto glucose medium to repress *EcoR*I and assay survival. Survival is expressed as the percentage of the cells plated that gave rise to colonies.


A
untreated
MMS(0.005%)
wild type
sir2Δ
sir2Δ hmlΔ
asf1Δ
asf1Δ
rad9Δ

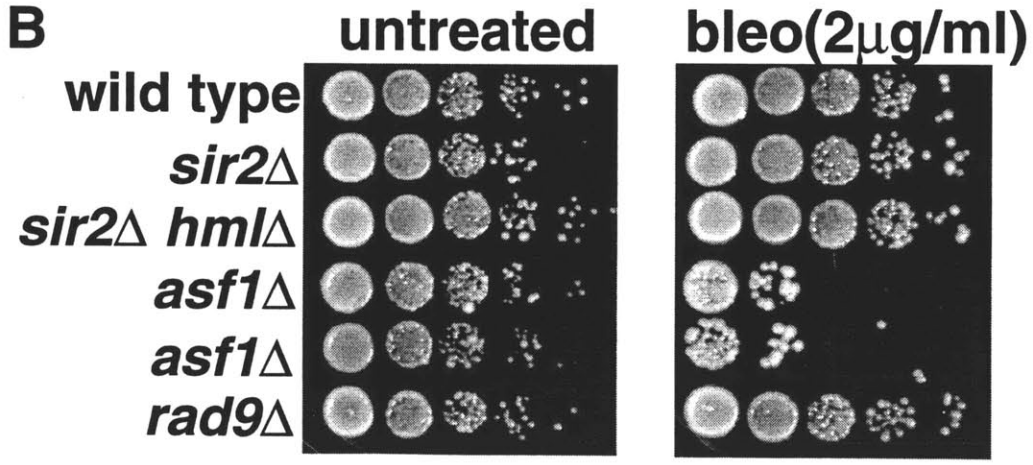
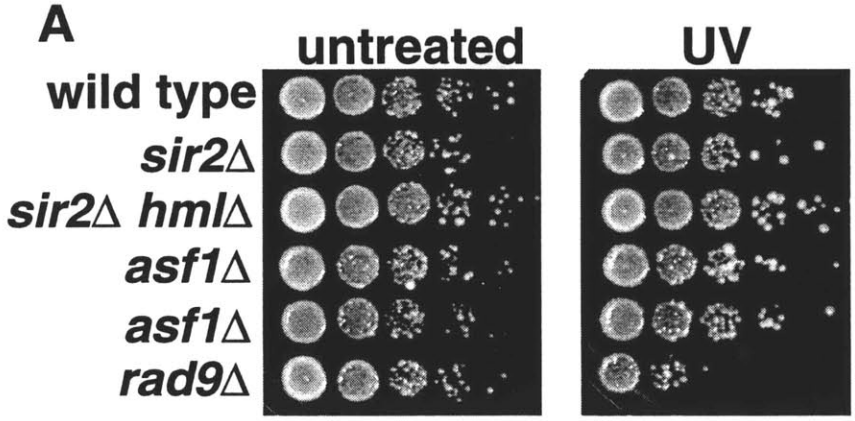

B
untreated
bleo(2μg/ml)
wild type
sir2Δ
sir2Δ hmlΔ
asf1Δ
asf1Δ
rad9Δ

FIGURE 5- Sensitivity of *ASF1* mutant cells to the DSB-inducing agents MMS and bleomycin. Cells of the indicated genotype were grown in YPD medium then plated in a 10-fold dilution series to YPD or YPD containing the indicated drug. (A). Cells plated onto YPD containing 0.005% MMS. (B). Cells plated onto YPD conatining 2μg/ml bleomycin sulfate.


A
untreated
UV
wild type
sir2Δ
sir2Δ hmlΔ
asf1Δ
asf1Δ
rad9Δ

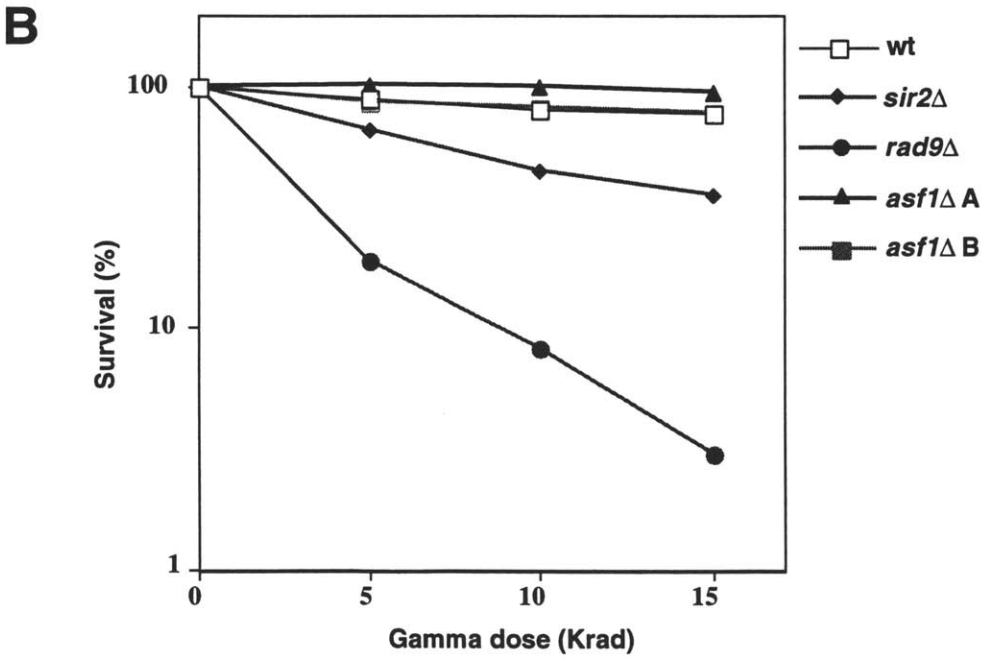

B
100
10
1
Survival (%)
0
5
10
15
Gamma dose (Krad)
wt
sir2Δ
rad9Δ
asf1Δ A
asf1Δ B

FIGURE 6- Sensitivity of *ASF1* mutant cells to UV- or γ-irradiation. (A). Cells of the indicated genotypes were grown in YPD then plated onto duplicate YPD media in 10-fold dilution series. One plate was untreated and the other was treated with 60 J/m^2 UV irradiation. (B). Wildtype, *sir2*Δ, *rad9*Δ, and *asf1*Δ cells were grown in YPD and treated with the indicated dose of γ-irradiation. Survival is expressed as the percentage of cells plated that gave rise to colonies.

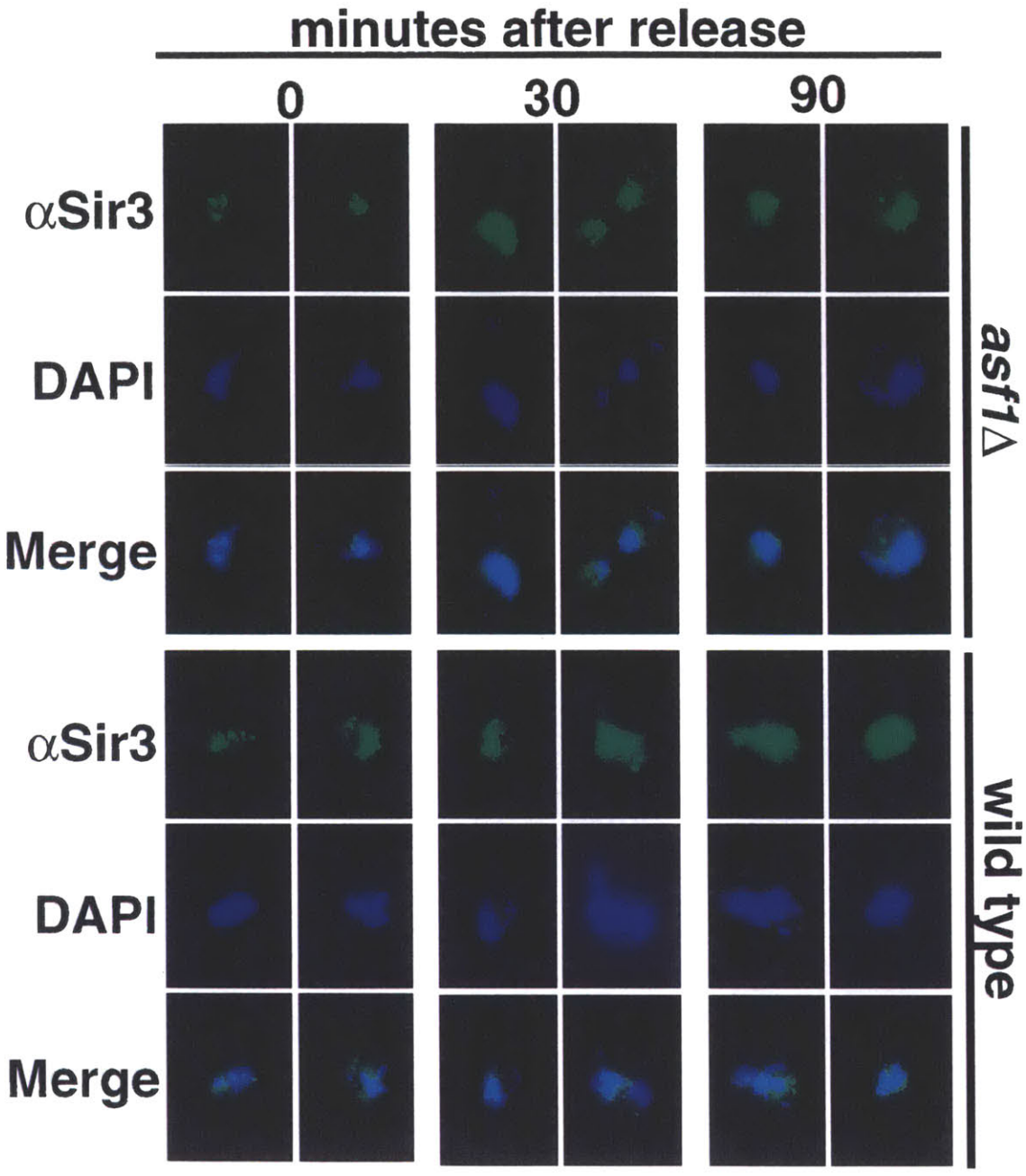

minutes after release
0
30
90
αSir3
DAPI
Merge
asf1Δ
αSir3
DAPI
Merge
wild type

FIGURE 7- Response of Sir3p to DSBs in *ASF1* mutant cells. Cells from *asf1*Δ or wildtype strains were arrested in G1 with α-factor, treated with 40 μg/ml bleomycin for 1 hour, and released into fresh medium. At 0, 30, and 90 minutes after release samples were fixed, spheroplasted, and stained with anti-Sir3p antibody. Redistribution of Sir3p following treatment with belomycin is indistiguishable for wildtype (bottom panels) and *asf1*Δ (top panels).

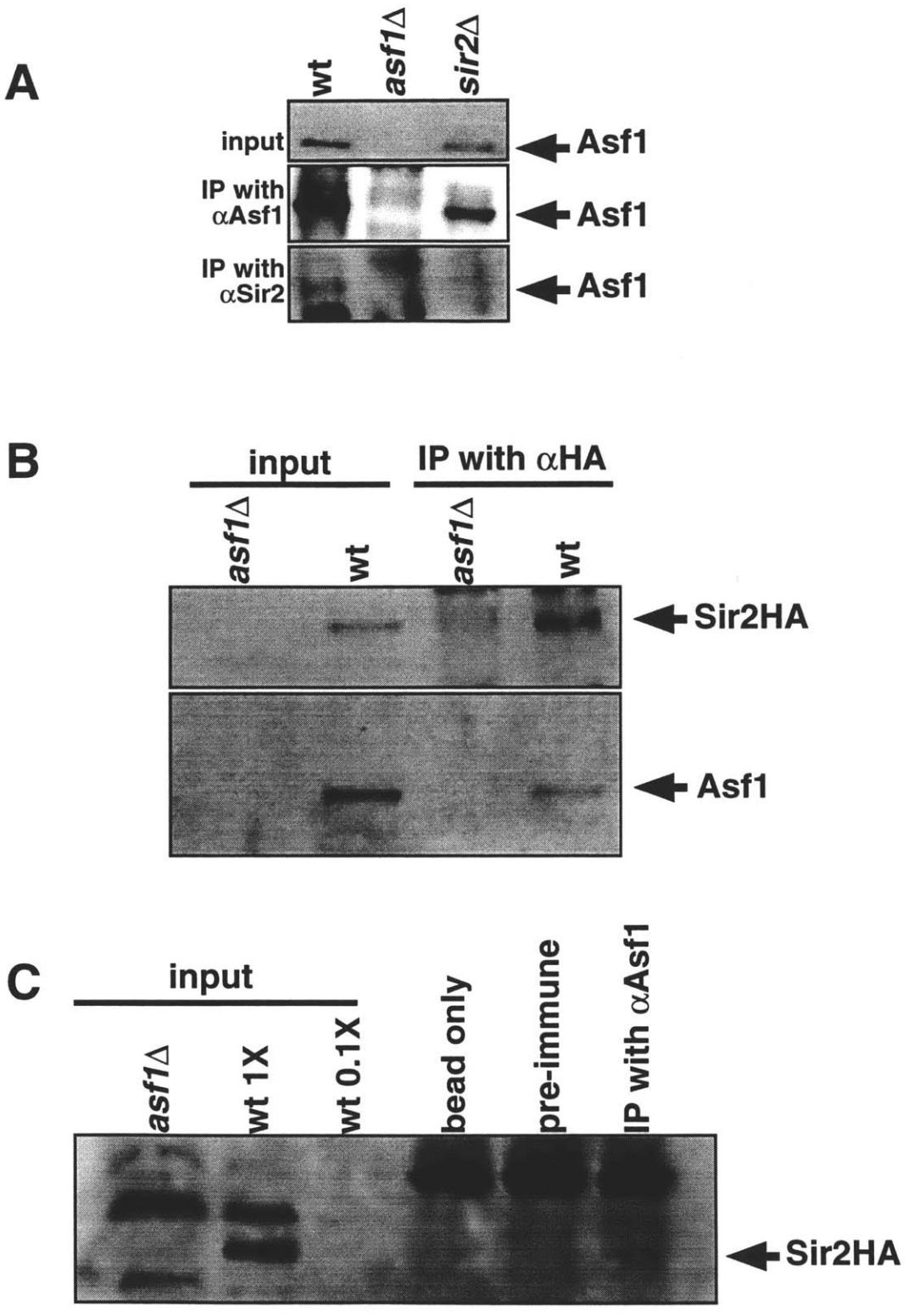

A
wt
asf1Δ
sir2Δ
input
IP with αAsf1
IP with αSir2
Asf1
Asf1
Asf1
B
input
IP with αHA
asf1Δ
wt
asf1Δ
wt
Sir2HA
Asf1
C
input
asf1Δ
wt 1X
wt 0.1X
bead only
pre-immune
IP with αAsf1
Sir2HA

FIGURE 8- Co-immunoprecipitation of Asf1p and Sir2p. (A). Western blots probed with affinity purified anti-Asf1p antibody. Top panel, whole cell extract from wildtype (lane 1), *asf1*Δ (lane 2), or *sir2*Δ (lane 3). Middle panel, immunoprecipitate from whole cell extract using anti-Asf1p antibody. Bottom panel, immunoprecipitate from whole cell extract using anti-Sir2p antibody from rabbit (gift of C. Armstrong). (B). Co-immunoprecipitation from whole cell extract containing a Sir2p-HA epitope tag. Lane1, whole cell extract from *asf1*Δ; lane 2, whole cell extract from wildtype; lane 3, immunoprecipitate from *asf1*Δ using anti-HA monoclonal antibody; lane 4, immunoprecipitate from wildtype using anti-HA monoclonal antibody. Top panel shows Western blot probed with anti-HA monoclonal antibody. Bottom panel shows Western blot probed with anti-Asf1p antibody. (C). Western blot probes with anti-HA monoclonal antibody. Lane 1, whole cell extract from *asf1*Δ; lane 2, 15μg whole cell extract from wildtype containing Sir2p-HA; lane 3, 1.5μg whole cell extract from wildtype containing Sir2p-HA; lane 4, precipitate prepared from Sir2p-HA extract using Protein A-speharose beads and no antibody; lane 5, precipitates prepared from Sir2p-HA extract using chicken pre-immune serum; lane 6, immunoprecipitate prepared from Sir2p-HA using anti-Asf1p antibody.

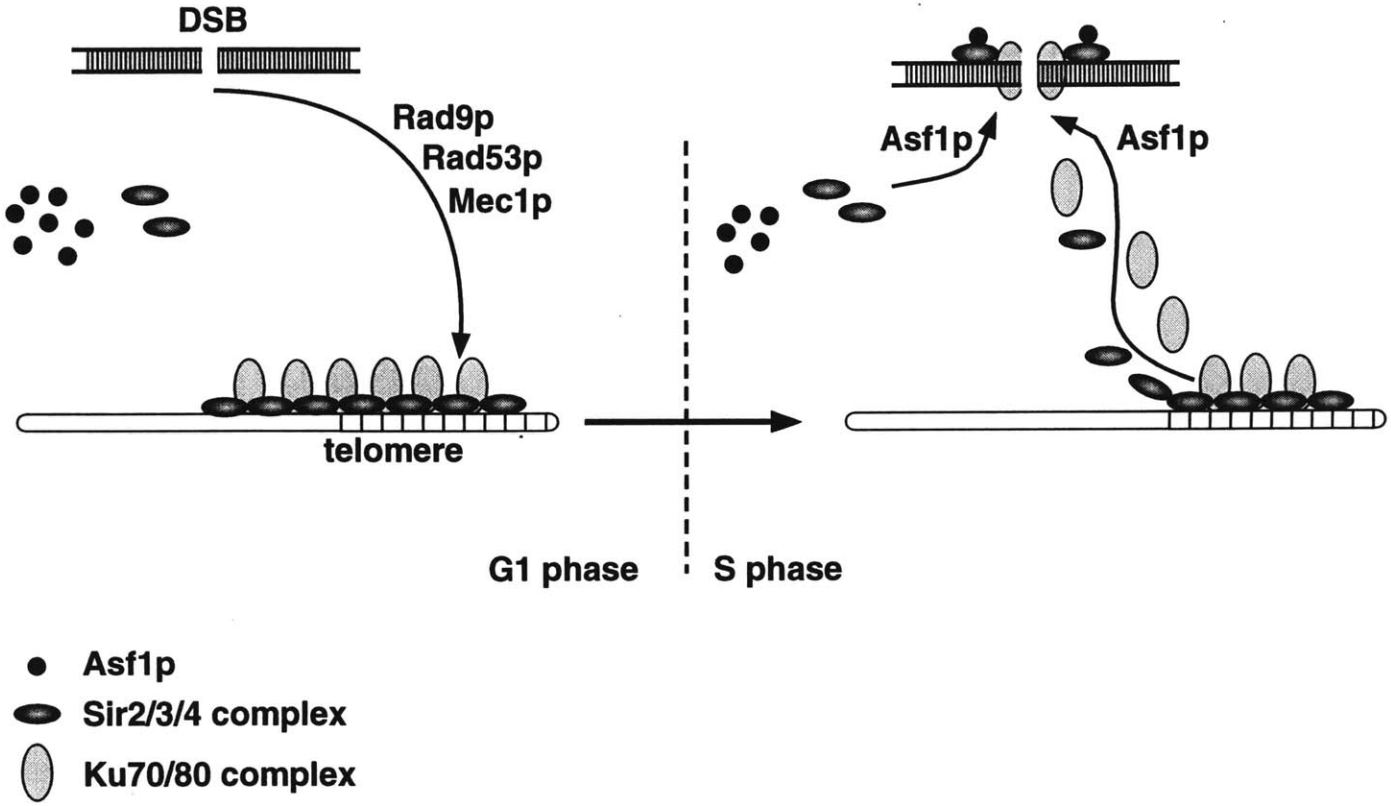

DSB
Rad9p
Rad53p
Mec1p
telomere
Asf1p
Asf1p
G1 phase
S phase
Asf1p
Sir2/3/4 complex
Ku70/80 complex

FIGURE 9- Model for role of Asf1p in response to DSBs. Following induction of DSBs the DNA damage checkpoint pathway signals telomeric proteins. During S phase this signal causes dissociation of telomeric Sir3p and Ku. Asf1p participates in recruitment of repair proteins to DSBs or in facilitating activity of repair proteins. In addition to the telomere-bound Sir complexes, there is a small pool of Sir proteins, not associated with the telomere, that does not require signaling by the checkpoint pathway for release, but does require Asf1p for subsequent steps in DNA repair.

Chapter 5: Summary and Conclusions

Recent research, leading in numerous directions, has provided a picture of highly dynamic chromatin structures that are involved in many processes within the nucleus. The budding yeast *Saccharomyces cerevisiae* has been a particularly fruitful model system in studying these various processes. Studies in yeast have illuminated the role of chromatin in transcriptional activation, transcriptional repression, mitotic and meiotic recombination, chromosome segregation, mating type switching, cell cycle checkpoint control, cellular aging, and DNA repair.

Silencing

One interesting function of yeast chromatin that has received much attention is silencing. Silencing was first recognized as an important constituent of yeast mating type control. Haploid yeast cells normally exist as either mating type **a** or mating type α. Cell mating type is determined by the mating type allele that is present at the *MAT* locus on chromosome III. A clue to the biology of yeast mating type came from the observation that chromosome III could sometimes be found as a circular chromosome (Strathern et al., 1979), evidently as a result of a recombination event between sequences on the left arm and sequences on the right arm of the chromosome (Klar et al., 1983). These recombining sequences were shown to contain complete copies of each of the mating type genes (mating type **a** usually on the right arm and mating type usually on the left arm) and were termed *HMR***a** and *HMLα* (Klar et al., 1980; Harashima and Oshima, 1980; Strathern et al., 1980; Nasmyth and Tatchell, 1980; Hicks et al., 1979). In addition, *HML* and *HMR* contain non-coding sequences that are homologous to one another, are not present at the *MAT* locus, and are required for transcriptional repression of the mating type genes (Brand et al., 1987;

Brand et al., 1985; Nasmyth and Tatchell, 1980; Strathern et al., 1980). This led to a model for mating type interconversion in which *HML* and *HMR* are transcriptionally silenced but serve as donors for gene conversion to the *MAT* locus during mating type switching. Subsequently numerous genes were identified that are required for transcriptional silencing of the *HM* loci.

Transcriptional silencing was also found to occur at or near telomeres (Gottschling et al., 1990). Genes that are inserted adjacent to telomeres are silenced, but frequently switch between silent and active transcriptional states. Telomere silencing requires many of the same genes necessary for *HM* silencing, including *SIR2, SIR3, SIR4,* and the repressor/activator gene *RAP1* (Aparicio et al., 1991). Telomeres were found to cluster near the nuclear periphery and co-localize with foci of these silencing proteins (Gotta et al., 1996). Mutations in genes that affect telomere silencing often also alter the structure or localization of telomeres (Cockell et al., 1995; Liu et al, 1994; hardy et al., 1992). These observations led to the model that telomeric heterochromatin plays a dual role: transcriptional silencing and structural maintenance.

A recent and surprising finding was that genes placed within the tandem array of ribosomal RNA genes on chromosome XII (rDNA) can also be silenced (Bryk et al., 1997; Smith and Boeke, 1997). In contrast to silencing at telomeres and *HM* loci, rDNA silencing requires *SIR2* and is antagonized by *SIR4*, but *SIR3* is dispensable (Smith et al., 1999; Smith et al., 1998; Smith and Boeke, 1997). Immunolocalization reveals that Sir2p concentrates in the nucleolus as well as at telomeres (Gotta et al., 1997). In the nucleolus Sir2p participates with Cdc14p and Net1p in a complex

(RENT complex) that is required for nucleolar silencing but not telomere or *HM* silencing (Straight et al., 1999; Shou et al., 1999).

DNA repair

Efficient and accurate repair of damaged DNA is essential to all cells since failure to properly repair damaged DNA can lead to mutagenesis, cellular transformation, or cell death. DNA double strand breaks pose a particular challenge for repair. Two general mechanisms have evolved in eukaryotic cells to repair DSBs: homologous recombination and nonhomologous end joining. Homologous recombination repairs a DSB by copying DNA from an intact homologous sequence or annealing complementary sequences flanking a break. DSBs are repaired by NHEJ when DNA from either side of a break is brought into juxtaposition and religated to form an intact DNA duplex.

Nonhomologous end joining in yeast requires *HDF1* and *HDF2*, encoding homologues of the mammalian DNA end binding factors Ku70 and Ku80 (Tskuamoto et al., 1996; Milne et al., 1996). NHEJ also requires the silencing genes *SIR2, SIR3,* and *SIR4*, providing a link between DSB repair and heterochromatin (Tsukamoto et al., 1997). In addition, NHEJ factors are required for normal telomere maintenance and heterochromatin formation (Boulton and Jackson, 1998; Gravel et al., 1998; Polotnianka et al., 1998). Mutation of *HDF1* or *HDF2* results in dispersal of clustered telomeres and a loss of telomere silencing (Laroche et al., 1998), while mutations in *MRE11* have no effect on silencing but result in shortened telomeres (Chamankhah and Xiao, 1999; Nugent et al., 1998). Together, these findings indicate that NHEJ may play important roles in chromosome

maintenance beyond repairing DSBs, and that these functions may be regulated by telomeric heterochromatin.

Screen for high-copy antagonists of silencing

To identify additional factors that can participate in or modify telomeric heterochromatin, a screen for high-copy antagonists of telomere silencing was performed. The dosage of the silent information regulators *SIR2, SIR3,* and *SIR4* was shown to be a key determinant of silencing not only at telomeres, but also *HM* loci and rDNA. These findings are consistent with other published reports that *SIR2* is a limiting component of silencing throughout the nucleus (Smith et al., 1998) and that silencing, especially at telomeres, is highly sensitive to the expression level of *SIR3* and *SIR4* (Gotta et al., 1998; Renauld et al., 1993).

In addition to the *SIR* genes, the previously identified modulators of silencing *SAS2* (Reifsnyder et al., 1996) and *NPL3* (Bossie et al., 1992) were identified. High copy *SAS2* weakly antagonized telomere and rDNA silencing and has been previously shown to differentially regulate *HML* and *HMR* silencing (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996), suggesting that *SAS2* encodes an accessory factor that may balance silencing between multiple loci. *NPL3* functions in nucleocytoplasmic transport and was identified in a screen for factors involved in *HM* silencing (Loo et al., 1995; Flach et al, 1994; Bossie et al., 1992) . Mutations in *NPL3* result in the accumulation of mRNA in the nucleus and invoke a stress response (Lee et al., 1996; Singleton et al., 1995). The role of *NPL3* in transcriptional silencing is not clear, but may be indirect and may reflect the responsiveness of heterochromatin to cellular stress.

One gene that strongly derepressed a telomeric marker when present in high copy number was *ASF1*, which was also identified as a high copy disruptor of silencing at *HML* (Le et al., 1997). *ASF1* expression is cell cycle regulated, reaching peak levels during S phase, and mutation of *ASF1* results in slight slow growth and hypersensitivity to the DNA alkylating agent, MMS (Le et al., 1997). These observations suggest that, in addition to silencing, *ASF1* might function in DNA repair or DNA synthesis. Here, *ASF1* is shown to be required for NHEJ, a role that is discussed in detail below.

The identification of a diversity of genes that can affect silencing, at telomeres, *HM* loci, and rDNA, confirms that silent heterochromatin in yeast is a dynamic element of the nucleus and is subject to modification by numerous processes. These findings further suggest that heterochromatin may be a central component of many different aspects of metabolism that involve DNA. One such aspect is the repair of DNA double strand breaks and is a major subject of the work presented here.

Role of *SIR* complex in NHEJ

The requirement for *SIR2, SIR3,* and *SIR4* in NHEJ suggested a role for heterochromatin in DSB repair (Tsukamoto et al., 1997). This was further supported by the identification of *ASF1* as an important determinant of silencing and MMS sensitivity. Changing environmental conditions can modify the strength of telomeric silencing, and Sir3p becomes phosphorylated in response to various stresses on the cell (Stone and Pillus, 1996). To determine if telomeric heterochromatin factors could respond to the presence of DNA damage, the distribution of Sir3p was determined

before and after treatment with various DNA damaging agents. Sir3p relocalizes from telomeres, showing a diffuse nuclear distribution, following induction of DSBs (Mills et al., 1999; Martin et al., 1999). This redistribution occurs specifically during the S phase of the cell cycle and requires the DNA damage checkpoint genes *MEC1* and *RAD9*.

The DNA damage checkpoint pathway is required for arresting the cell cycle when DNA damage is detected, and enforcing that arrest until the damage is repaired (reviewed in Weinert, 1998). Elements of this pathway are also utilized to halt cell cycle progression until DNA replication is complete. In addition to cell cycle arrest, the checkpoint machinery activates transcription of DNA repair genes when damage is detected (reviewed in Weinert, 1998). The results in Chapter 3 provide the first demonstration that the checkpoint machinery can also signal to pre-formed DNA repair machinery that is sequestered at telomeres. Signaling to factors resident at telomeres is a rapid mode of response to DNA lesions. This response is advantageous because it allows the cell to repair DNA damage using pre-existing repair factors that are poised to respond immediately.

It was also shown that, following induction of DSBs, Sir3p dissociates from telomeres and becomes associated with DNA near sites of damage (Mills et al., 1999). The yeast Ku70 end binding factor, which also resides at telomeres in undamaged cells, also arrives at sites of damage following induction of breaks (Martin et al., 1999). These findings demonstrate that telomeres act as reservoirs for proteins that are needed elsewhere in the genome under special circumstances.

The function of Ku and Sir proteins at DNA breaks is unknown. One possibility is that these factors collaborate at DSBs to form repressive

heterochromatin that protects the ends against nucleolytic degradation or other inappropriate enzymatic processing. Another possibility is that Ku or the Sir complex at DSBs stimulates the activity of repair factors such as DNA ligase IV to accelerate repair of the lesion. Since relocalization of telomeric proteins to DSBs occurs during S phase, a third possibility is that broken ends must be isolated from DNA replication machinery until the break can be repaired.

Role of *ASF1* in NHEJ

In addition to the Sir complex and the Ku heterodimer, NHEJ requires a variety of other accessory factors. These include the Xrs2p/Rad50p/Mre11p complex (Tsukamoto et al., 1997; Moore and Haber 1996) and DNA ligase IV (Teo and Jackson, 1997; Schar et al., 1997; Wilson et al., 1997) in yeast and the DNA-PK_{cs} in mouse and human (reviewed in Lieber et al., 1997). The antisilencing factor Asf1p is shown here to be required for NHEJ, and to interact in a complex with Sir2p. Deletion of *ASF1* results in increased telomere silencing and a complete loss of NHEJ efficiency. In contrast, mutations in *RAD9, MEC1,* or *RAD53*, that prevent release of Sir3p from telomeres in response to DSBs (Mills et al., 1999; Martin et al., 1999), result in only a 2-fold decrease in end-joining efficiency. Interestingly, mutation of *ASF1* does not affect the dissociation of Sir3p from telomeres following induction of DSBs. These observations indicate that Asf1p acts at a step downstream of release in the Sir-dependent repair pathway, and may be involved in recruitment or priming of the Sir complex for DSB repair.

Model for heterochromatin response to DSBs

The findings presented here have led to the current model for the response of telomeric heterochromatin to DSBs (Chapter 4 Figure 9). Breaks incurred before cells enter into S-phase are detected by the known damage sensing apparatus. Upon entry into S-phase, the DNA damage checkpoint signaling pathway communicates with telomeres and stimulates release of a complex of Sir proteins, the Ku end-binding heterodimer, and possibly other repair factors. These factors are released beginning from the most centromere-proximal extent of heterochromatin polymerization and release progresses outward toward the end of the chromosome. The newly released complexes, in conjunction with a small, non-telomeric pool (see Chapter 4), are recruited into chromatin at or near DSBs to facilitate repair. After DSBs have been repaired and cells exit S-phase, Sir and Ku proteins return to telomeric foci. Formation of Sir-dependent heterochromatin around DNA breaks may be required during S-phase to prevent inappropriate processing, recombination, or passage of a replication fork through the DSB.

Implications and Questions

This work clearly demonstrates an involvement of yeast heterochromatin factors in the repair of DSBs. However, many features of the response to DSBs remain obscure. Sir3p dissociates from telomeres specifically during S-phase, but the importance of this cell cycle specificity and how it is conferred are not known. One candidate for a regulator of the S-phase specificity Asf1p since its expression oscillates during the cell cycle, reaching maximum levels during S phase. It will also be interesting to

determine if other factors assemble into chromatin near DSBs, and if so, what is the role of DSB-heterochromatin in the repair of the lesion?

Saccharomyces cerevisiae has been a useful system for the study of NHEJ. The involvement of chromatin factors and the importance of the Xrs2p/Rad50p/Mre11p nuclease complex were first recognized in yeast. However, NHEJ has also been well characterized in mammals and is important in immunological and neurological development as well as DNA repair (Frank et al., 1998; Gao et al., 1998; Gu et al., 1998; Gu et al., 1997). Many human syndromes also involve defects in DSB repair. These include ataxia telangiectasia (Pandita and Hittelman, 1992), Nijmegen breakage syndrome (Carney et al., 1998; Varon et al., 1998), and certain types of cancers. Therefore, an understanding of NHEJ may provide insight into human diseases arising from faulty DNA repair and may suggest new therapies for the treatment of such diseases.

There is striking conservation between yeast and human in many DNA repair proteins, suggesting that the basic mechanisms of DNA repair are conserved among different species. Recent work from several laboratories has led to the identification of human homologues of the yeast silencing factor Sir2p. Perhaps some of the mechanisms for forming and regulating heterochromatin are also conserved between yeast and mammals. It will therefore be of interest to identify functional mammalian homologues of other yeast chromatin factors and determine their role in the repair of DSBs. The yeast DSB repair mechanism achieves a rapid response by signaling, pre-existing, telomere-bound proteins using the DNA damage checkpoint pathway. Hints from the pathology of A-T suggest that a similar system may function in mammalian cells.

The recent work on NHEJ in *Saccharomyces cerevisiae* has provided new insights into the general mechanisms of DSB repair and has revealed previously unknown complexity. Furthermore, this work has demonstrated the central role that heterochromatin factors fulfill in DNA repair, transcriptional silencing, and telomere maintenance. The knowledge gained from the study of NHEJ in yeast may also shed new light on the mechanisms of this important DNA repair pathway in mammalian cells.

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